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Incorporation of [1-^{14}C] and [3-^{14}C] butyrate into milk constituents by the perfused cow's udder

By MONIQUE LAURYSSENS, R. VERBEKE, G. PEETERS and M.-THÉRÈSE REINARDS

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(*Received* 16 *September* 1959)

Summary. One half-udder from a lactating cow was perfused for 110 min with blood containing 1 mc of [1-^{14}C]butyrate. Inactive sodium butyrate was added at the eightieth minute. The volume of milk collected was 320 ml. One half-udder from another cow was perfused for 120 min in the presence of 0·5 mc [3-^{14}C]butyrate, inactive sodium acetate being added at the sixtieth minute. The volume of milk collected was 150 ml.

The total amount of $^{14}CO_2$ recovered was 5·7 % of the [1-^{14}C]butyrate given and 7·6 % of the [3-^{14}C]butyrate given. In both instances casein, followed by the volatile fatty acids, showed the highest specific activity of the constituents isolated from the milk. The glyceride fatty acids in the udder were fifty times as active as those in the milk. With [3-^{14}C]butyrate the activity of the total fatty acids amounted to 24 % and that of total lactose to 0·38 % of the added ^{14}C. Butyrate did not appear to be used for glycogenesis in the perfused gland. The specific activity of the lower fatty acids of both the udder and the milk increased stepwise with increasing chain length to reach a maximum at C_{10}. A satisfactory explanation for this peculiar ^{14}C distribution cannot be given at the present time. There was no evidence of direct esterification of butyrate. Most of the activity of casein was due to labelled glutamic and aspartic acids, the activity of the former being four times as high as that of the latter. The acids of the Krebs cycle isolated from the udder tissue when [3-^{14}C]butyrate was given showed very high activity. No striking differences were observed between the results of the two experiments. It is concluded that butyrate is split into two C_2 components which behave identically. These are utilized for fatty acid synthesis and take part in the Krebs cycle. The relative ^{14}C distribution between the components isolated from milk and those from tissue may be a reflexion of the secretory processes in the udder cells, synthesized fat tending to be secreted in the alveoli after the other constituents.

Butyric acid is a product of fermentation of food in the intestinal tract of various animals especially in the rumen of cattle and sheep. Typical mixtures of volatile fatty acids, isolated from peripheral blood from cows, contained on a molecular basis mean values of 93·3 % acetic acid, 2·39 % propionic acid and 2·51 % butyric acid (McClymont, 1951). From these and other data one may estimate that 1 l. of arterial

blood of a cow may contain on the average 1·46 mM of acetate and 0·04 mM of butyrate. Taking into account the important role of acetate as a precursor of the volatile fatty acids of milk in the ruminant it is likely that butyrate will also be utilized for this purpose. Popják, French, Hunter & Martin (1951) studied the synthesis of milk volatile fatty acids in the lactating goat and suggested that a C_4 component derived from the blood probably played an important part in this process. According to these authors β-hydroxybutyrate could be this C_4 component. Intact dairy cows were injected with sodium [1-^{14}C] and [2-^{14}C]butyrate by Kleiber *et al.* (1954). About 6% of the [1-^{14}C] and 22% of the [2-^{14}C] of the butyrate given were recovered in the organic constituents of the milk. In contrast to acetate more of both the [1-^{14}C] and the [2-^{14}C] of the butyrate given appeared in the lactose and casein than in the fat of the milk. Peeters, Coussens & Sierens (1953) studied the metabolism of inactive butyrate in the perfused cow's udder. After addition of butyrate, traces of acetoacetate were detected in the perfusion blood. Oxygen uptake was higher but the respiratory quotient (R.Q.) values were lower in the butyrate half than in the control half of the udder. Addition of acetate resulted in a highly increased respiration with R.Q. values always well above unity. It was concluded that butyrate, in contrast to acetate, was not one of the main precursors of milk fat in the isolated cow's udder.

In the experiments described here a study has been made of the metabolism of [1-^{14}C] and [3-^{14}C]butyrate by the perfused udder. Some of the results of the [1-^{14}C]butyrate experiment have been reported previously in a preliminary form (Lauryssens, Verbeke & Peeters, 1957).

EXPERIMENTAL

Perfusion of half-udders

The metabolism of each labelled precursor was studied on one perfused half-udder. Half-udders from different cows were used. The perfusion technique was that described by Peeters & Massart (1947). Cows with a normal lactating udder, free from mastitis, were selected in the slaughterhouse. Oxytocin was injected and the udder milked as completely as possible just before the cow was shot. The udder was then removed as quickly as possible and transported to the laboratory. The glands were bisected along the median septum and one-half was used in the perfusion experiment, the other half being discarded. The half-udder was perfused at constant pressure with 8·5 l. oxygenated, heparinized cow's blood at 38 °C. At the end of the experiment oxytocin was injected into the artery and milk was collected, the teats being cannulated. The collection of $^{14}CO_2$ during the perfusion was performed as described previously (Verbeke, Lauryssens, Peeters & James, 1959).

The metabolism of [1-^{14}C]butyrate was studied on one half-udder from a cow yielding 16 l. milk daily. Sodium [1-^{14}C]butyrate (1 mc = 110 mg) dissolved in 50 ml saline was added to the blood at the fifteenth minute after the perfusion began. Eighty minutes later the addition of a solution of 5 g inactive sodium butyrate in 200 ml water by means of a constant drip device was begun. The perfusion experiment was stopped 110 min after the addition of the labelled precursor and 320 ml milk were collected.

[3-^{14}C]Butyrate was studied in a half-udder from a cow yielding 15 l. milk daily. Sodium [3-^{14}C]butyrate (0·5 mc = 33 mg) was dissolved in 50 ml saline and added to the blood at the fifteenth minute of perfusion. One hour later the continuous addition of a solution of inactive $CH_3COONa.3H_2O$ (5·25 g in 250 ml water) was started. The experiment was stopped 2 h after adding the labelled precursor and 120 ml milk were collected.

[1-^{14}C]Butyrate was obtained from the Radiochemical Centre, Amersham, and [3-^{14}C]butyrate from the Centre d'Etudes Nucléaires, Saclay.

Isolation of milk proteins and casein amino acids

Casein was isolated from the skim-milk by isoelectric precipitation (pH 4·6). The whey proteins were precipitated from the filtrate by addition of an equal volume 20% (w/v) trichloroacetic acid (Verbeke *et al.* 1959). The casein samples were refluxed for 36–48 h in 300 volumes 6 N-HCl. The amino acids were isolated from these hydrolysates using a combination of chromatography on ion-exchange columns (Hirs, Moore & Stein, 1954) and starch columns (Stein & Moore, 1948) as previously described (Verbeke *et al.* 1959). The isolated amino acids were checked for purity by paper chromatography.

Isolation of lipid fractions from tissue and milk, followed by chromatographic separation of glyceride fatty acids

Total fat was extracted from tissue and milk and purified from contaminating [1-^{14}C] or [3-^{14}C]butyrate. Phospholipids and neutral fat were isolated. From the neutral fat fraction, glycerol, cholesterol, steam volatile and steam non-volatile fatty acids were prepared. The non-volatile long chain fatty acids were separated into a solid and a liquid fraction by the lead salt method (Twitchell, 1921); the liquid fraction was not further examined. From the steam volatile fraction the C_4 and C_6 acids were isolated by gas—liquid chromatography (James & Martin, 1952), and the acids from C_8 to C_{16} were obtained by reversed phase partition chromatography (Howard & Martin, 1950). From the solid higher fatty acid fraction myristic, palmitic and stearic acids were isolated by the reversed phase technique already mentioned. Tissue fatty acids only were examined chromatographically in the [3-^{14}C]butyrate experiment. The experimental procedure used has been described previously (Verbeke *et al.* 1959).

Isolation of lactose

In both experiments lactose was isolated from whey as described by Reiss & Barry (1953). Isolation of lactose from udder tissue was performed only in the [3-^{14}C]-butyrate experiment. Udder tissue (400 g) was homogenized in 2 volumes of ice-cold water and the mixture centrifuged after extraction for 2 h. Fat was removed from the supernatant liquid by repeated extractions with light petroleum. The water layer was deproteinized with $ZnSO_4$ and $Ba(OH)_2$ and desalted by passage through a mixed bed ion-exchange column (Amberlite IR-4B, OH^-; Amberlite IR-120, H^+). After concentration of the percolate *in vacuo* to a final volume of 10 ml, the lactose was adsorbed on to a Norit column (Whistler & Durso, 1950). The lactose eluate was concentrated to 100 ml and freeze-dried. Lactose was crystallized from the residue

(Reiss & Barry, 1953). All the lactose samples isolated were chromatographically pure.

Lactose was estimated in milk and udder tissue by the method of Folley & Greenbaum (1947). The percentage of the added ^{14}C dose which was recovered in the lactose fraction from the [3-^{14}C]butyrate experiment was calculated from the total lactose content and from the specific activities of the lactose isolated from the tissue and the milk.

Isolation of organic acids from udder tissue

Citric, lactic, succinic, malic and fumaric acids were isolated in the [3-^{14}C]butyrate experiment from 500 g of ground udder tissue which had been stored for a few months under ethyl alcohol at $-15°C$.

Citric acid was isolated by chromatography on Dowex 1 and silica-gel as described previously (Verbeke *et al.* 1959). For the isolation of the other acids, the method described previously was modified. The lactic acid peak emerging from the Dowex 1 $H.CO_2^-$ column was freed from the bulk of formic acid by steam distillation. The residue of the distillate was made alkaline and boiled for 10 min. The lactate solution was concentrated to dryness, acidified and freed from formic acid by chromatography on an ether Celite column (Phares, Mosbach, Denison & Carson, 1952). The fractions eluted on the Dowex 1 column between lactic acid and citric acid were combined, evaporated to dryness and applied to the top of a silica-gel column (Bulen, Varner & Burrell, 1952). Succinate was eluted in 15% (v/v) butanol—chloroform, and malic acid in 35% (v/v) butanol—chloroform. Sixty fractions of the effluent of the Dowex 1 column, emerging after the citric-acid peak, were combined and concentrated *in vacuo* to dryness. By chromatography of the residue on a silica-gel column, fumaric acid was eluted together with traces of formic acid in 15% (v/v) butanol—chloroform (Bulen *et al.* 1952). Fumaric acid was freed from contaminating formic acid by passage through an ether Celite column (Phares *et al.* 1952). All the acids isolated were quantitatively determined by titration of the peaks. The identity and purity of each organic acid was checked by paper chromatography (Kalbe, 1954).

Radioactivity measurements

The methods of plating and counting of the soaps, amino acids and organic acids have been described earlier (Verbeke *et al.* 1959).

Samples of lactose, glyceryl trinitrobenzoate, phospholipids, cholesterol digitonide and proteins were combusted and the resultant CO_2 was trapped as $BaCO_3$ which was assayed for ^{14}C.

In the tables the specific activities are recorded as μmc together with the S.D. of the mean from at least triplicate samples.

RESULTS

Carbon dioxide

Fig. 1 shows the specific activity of the expired CO_2, expressed as a percentage of the added dose, plotted against the time that had elapsed after administration of the labelled substrates. Relatively little $^{14}CO_2$ was collected during the first minutes of

perfusion, but its production increased progressively during the experiment and reached a maximum near the end of the perfusion. In all in the [1-^{14}C] and [3-^{14}C]-butyrate experiments 5·7 and 7·6% of the added ^{14}C were collected, respectively.

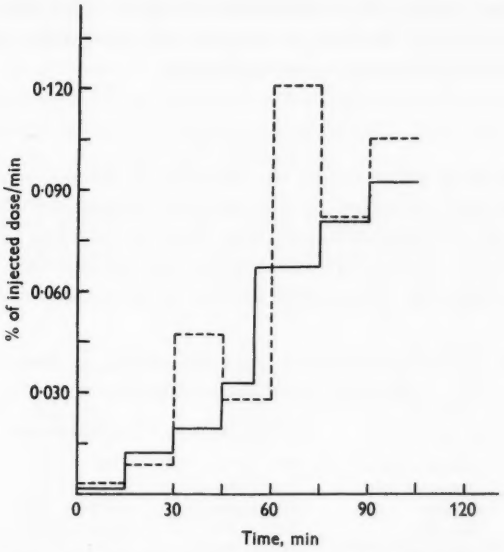

Fig. 1. Specific activity of $^{14}CO_2$, expressed as percentage of added dose, eliminated by the artificial lung system during perfusion experiments. —— After administration of [1-^{14}C]butyrate. - - - - - - After administration of [3-^{14}C]butyrate.

Table 1. *Specific activities* (μmc/mg *carbon*) *of the crude fractions isolated from udder tissue and milk*

	[1-^{14}C]Butyrate, 1 mc added		[3-^{14}C]Butyrate, 0·5 mc added	
Fraction isolated	Tissue	Milk	Tissue	Milk
Volatile fatty acids	4·8 ±0·1	0·10 ±0·01	4·0 ±0·1	—
Non-volatile fatty acids	1·10±0·05	0·00	0·81±0·03	—
Glycerol	0·29±0·02	—	—	—
Phospholipids	0·97±0·05	—	0·45±0·04	—
Cholesterol	—	—	0·14±0·01	—
Lactose	—	0·079±0·005	0·23±0·02	0·05±0·02
Casein	—	1·11 ±0·04	—	1·32±0·04
Whey proteins	—	0·31 ±0·02	—	0·43±0·02

Where no figure is given no determination was made.

Crude fractions

The activities of the crude fractions are summarized in Table 1. Both [1-^{14}C]-butyrate and [3-^{14}C]butyrate behaved in the same way. A similar ^{14}C distribution pattern was observed in previous work after perfusion in the presence of [1-^{14}C]-isovalerate (Verbeke *et al.* 1959) and of [1-^{14}C]caproate (Lauryssens, Verbeke, Peeters & Donck, 1959). Of the milk substances casein was the most active fraction, followed

by the whey proteins, the volatile glyceride fatty acids and finally the lactose. Of the udder fractions the volatile glyceride fatty acids showed the greatest incorporation of ^{14}C, followed by the higher glyceride fatty acids and the phospholipids. It should be mentioned that casein from udder tissue was not isolated. Some activity was also detected in glycerol, lactose and cholesterol isolated from udder tissue. In the [3-^{14}C]butyrate experiment the lactose of both milk and udder tissue was isolated, the latter being four times as active as the former.

Amino acids

There was no striking difference in the labelling of the non-essential amino acids in the two experiments. Glutamic acid showed the highest specific activity followed by aspartic acid, and was approximately four times as active as aspartic acid in both experiments (Table 2). Serine, glycine, alanine and proline were labelled only to a slight extent. The essential amino acids showed no detectable radioactivity.

Table 2. *Specific activities* (μmc/mg *carbon*) *of amino acids isolated from casein hydrolysates*

	[1-^{14}C]Butyrate	[3-^{14}C]Butyrate
Glutamic acid	6·1 ±0·6	7·84 ±0·08
Aspartic acid	1·5 ±0·4	2·01 ±0·02
Serine	0·25±0·08	0·496±0·005
Glycine	0·12±0·04	0·101±0·005
Alanine	0·05±0·02	0·090±0·005
Proline	0·06±0·02	0·07 ±0·01

Fatty acids

In Table 3 the specific activities of the glyceride fatty acids are recorded. The acids showed, in both experiments, a stepwise increase in specific activity with the elongation of the chain from butyric up to decanoic acid, followed by a stepwise decrease up to palmitic acid. Extremely little activity was found in stearic acid. The udder and milk fatty acids showed the same ^{14}C distribution pattern. In the [1-^{14}C]butyrate experiment the udder fatty acids had activities that were some fifty times greater than those of the corresponding milk acids. The fact that the udder fatty acids had higher activities than the milk fatty acids had already been observed in previous experiments (Verbeke *et al.* 1959; Lauryssens *et al.* 1959; Cowie *et al.* 1951).

Table 3. *Specific activities* (μmc/mg *carbon*) *of the glyceride fatty acids*

	[1-^{14}C]Butyrate		[3-^{14}C]Butyrate
	Udder tissue	Milk	Udder tissue
Butyric	4·27 ±0·18	0·082±0·009	2·65 ±0·09
Hexanoic	5·43 ±0·23	0·096±0·010	5·01 ±0·18
Octanoic	7·53 ±0·56	0·108±0·009	5·91 ±0·15
Decanoic	8·04 ±0·34	0·163±0·014	6·38 ±0·16
Lauric	7·73 ±0·40	0·120±0·013	5·87 ±0·17
Myristic	4·02 ±0·21	0·070±0·007	2·39 ±0·12
Palmitic	1·04 ±0·06	0·00	0·79 ±0·02
Stearic	0·143±0·010	0·00	0·045±0·005

Organic acids

From 500 g of udder tissue, which had been stored for a few months under ethyl alcohol at −15 °C, the isolated organic acids (citric, malic, fumaric, succinic and lactic acids) amounted to a total of 907 μmoles. The relative distribution of the organic acids, expressed in molar percentages, is given in Table 4. In the [3-^{14}C]-butyrate experiment the acids of the Krebs cycle showed the highest specific activity of all the compounds isolated and all of them had specific activities of the same order (Table 4). The specific activity of lactic acid, however, was only one-sixth or one-seventh of the activity of the Krebs cycle acids.

Table 4. *Relative distribution and specific activities of organic acids isolated from udder tissue in the* [3-^{14}C]*butyrate perfusion experiment*

	Distribution molar percentage	Specific activity μmc/mg carbon
Citric acid	18·1	21·2 ±0·2
Succinic acid	20·1	20·4 ±0·2
Fumaric acid	2·45	17·5 ±0·2
Malic acid	5·95	18·7 ±0·2
Lactic acid	53·4	3·15±0·03

DISCUSSION

More of the labelled C_1 and C_3 atoms of the butyrate appeared in the fat than appeared in the lactose. In the [3-^{14}C]butyrate experiment 24 % of the added ^{14}C was recovered in the total glyceride fatty acids while only 0·37 % was recovered in the lactose. Casein could not be included in these balance studies, since the specific activity of the casein in the udder tissue was not known. Low specific activities were observed in lactose, alanine, serine and glycine. An appreciable amount of labelling was found in the lactic acid from udder tissue, but this activity was much below that observed in the Krebs cycle acids. It can be estimated that in the [1-^{14}C]butyrate experiment approximately 14 % of the added ^{14}C activity appeared in the total glyceride fatty acids. It would appear that butyrate was not being used for glycogenesis in the perfused udder. This conclusion is in sharp contrast with that reached with the intact cow by Kleiber *et al.* (1954) who noted that an important amount of ^{14}C from labelled butyrate was incorporated in the lactose of milk. It may be that some organ other than the udder is involved in the conversion of butyrate to carbohydrates in the intact cow. Evidence that a reaction of this kind may occur in the liver of ruminants was obtained recently in experiments by Shaw, Lakshmanan, Chung, Leffel & Doetsch (1958) in which goat liver was perfused.

The specific activities of the fatty acids isolated from udder tissue were 10 to 100 times higher than those of the fatty acids from milk in all the present perfusion experiments. The activities of lactose, however, were of the same order in both milk and udder tissue. Although no information is available about the activity of casein in tissue, it is possible that casein would behave like lactose and show activities of the same order both in udder tissue and in milk. Precursors labelled with ^{14}C were injected unilaterally by Wood, Joffe, Gillespie, Hansen & Hardenbrook (1958) into

the pudic artery which supplies blood to one-half of the cow's udder. The maximum specific activities of the glycerol and fatty acids of the milk from the injected side were reached after a considerably longer time (4 h) than they were reached in the casein or the lactose (1–2 h). Wood and his colleagues suggested that these differences might be the result of different secretory processes, the secretion of fat in the alveoli, after its synthesis in the cells, perhaps taking place more slowly than that of lactose or casein. The distribution of ^{14}C between milk and tissue components in the present perfusion experiments may be explained on this basis, the perfusion being stopped 2 h after the administration of the labelled precursor.

The C_1 and C_3 atoms of butyrate are incorporated less efficiently by the perfused udder into fatty acids than is the C_1 atom of acetate. The rate of $^{14}CO_2$ production was highest with [1-^{14}C]acetate. It appears that acetate is utilized to a higher degree than butyrate in the cow's udder, an observation that agrees well with conclusions reached previously in gas exchange experiments (Peeters *et al.* 1953). The results obtained are most readily explained by assuming that in the udder butyric acid is split into its two C_2-components. Both these components must then behave in the same way, since no striking differences were observed between the results with the [1-^{14}C] and [3-^{14}C]butyrates. From the high activities observed in the organic acids from udder tissue in the [3-^{14}C]butyrate experiment it may be concluded that the C_2-components are metabolized by the Krebs cycle. The important activities found in the glutamic and aspartic acids, which were obtained by acid hydrolysis of the casein, can easily be explained on the basis of this cycle. In experiments with both butyrates, glutamic acid showed the highest activity. The same pattern of labelling of amino acids has been observed previously in perfusion experiments carried out in the presence of [1-^{14}C]acetate (Verbeke, Aqvist & Peeters, 1957), [1-^{14}C]caproate (Lauryssens *et al.* 1959) and [1-^{14}C]isovalerate (Verbeke *et al.* 1959). This ^{14}C distribution pattern is not the result of $^{14}CO_2$ fixation and differs from the one observed by perfusion in the presence of [1-^{14}C]propionate, where the highest activity was found in aspartic acid, followed by glutamic acid (Verbeke *et al.* 1957). The activities observed in lactic acid, lactose, alanine, glycine and serine can be explained by passage of ^{14}C from the Krebs cycle by way of oxaloacetate and pyruvate to the glycolytic pathway. The C_2 units, derived from butyrate, participate also in the synthesis of the volatile fatty acids of milk up to and including palmitic acid. Once more these results indicate the capacity of the mammary gland to synthesize the lower milk fatty acids, the C_{18} acids being supplied probably from the bloodstream.

The specific activity of the volatile fatty acids increased stepwise from butyric acid to caprylic acid and then decreased gradually. A similar picture was obtained after intravenous injection of a lactating goat with [1-^{14}C]acetate (Popják *et al.* 1951) and after perfusion of the isolated cow's udder in the presence of [1-^{14}C]isovalerate (Verbeke *et al.* 1959) and [1-^{14}C]caproate (Lauryssens *et al.* 1959). Kumar, Lakshmanan & Shaw (1959) observed a gradual increase in the specific activity (counts per min per μmole) of the milk fatty acids from C_4 to C_8 after perfusion in the presence of β-hydroxybutyrate. After perfusion in the presence of [1-^{14}C]acetate, however, the specific activity of the volatile fatty acids of the udder was found to decrease with increasing chain length (Cowie *et al.* 1951).

From degradation results from butyric and caproic acids, derived from their

acetate experiment on the living goat, Popják *et al.* (1951) concluded that butyric acid in the milk has a dual origin, 40 % being synthesized in the udder from acetate and 60 % being derived from a C_4 compound, which is absorbed from the bloodstream. According to these authors, β-hydroxybutyrate fulfills the requirements of this additional precursor of milk butyric acid.

Kumar *et al.* (1959) explained their results obtained after perfusion in the presence of β-hydroxybutyrate in the following way. β-Hydroxybutyrate would be reduced to butyrate, which would be converted directly to glycerides. The C_2 moieties of β-hydroxybutyrate would not be utilized identically; the carboxyl end of the molecule would be used preferentially for lipogenesis. If the carboxyl end of the β-hydroxybutyrate underwent very rapid exchange with the C_2 pool—which is negligible in size in the perfused udder—then highly active C_2 compounds would be produced which would bring about the elongation of the C_4 chain. In this way the newly synthesized C_6, C_8, C_{10} acids would show an increase in specific activity.

The present results, however, do not support these hypotheses. While they strongly indicate that butyric acid is broken down into C_2 units, no experimental evidence was obtained to indicate that butyrate was converted directly to glycerides. A similar ^{14}C distribution pattern in the volatile fatty acids has been observed by the present authors in perfusion experiments carried out in the presence of different precursors (Verbeke *et al.* 1959; Lauryssens *et al.* 1959). It seems probable that by direct esterification a much higher activity would have been observed in the C_4 chain. It is clear also that both C_2 moieties of butyric acid were utilized identically. It should be made clear that the present results do not entirely contradict the theories of Popják *et al.* (1951) and Kumar *et al.* (1959) that have just been mentioned, since butyric acid, added to the circulating blood, does not necessarily behave in the same way as does butyric acid derived in the udder from β-hydroxybutyrate.

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REFERENCES

Bulen, W. A., Varner, J. E. & Burrell, R. C. (1952). *Anal. Chem.* **24**, 187.
Cowie, A. T., Duncombe, W. G., Folley, S. J., French, T. H., Glascock, R. F., Massart, L., Peeters, G. & Popják, G. (1951). *Biochem. J.* **49**, 610.
Folley, S. J. & Greenbaum, A. L. (1947). *Biochem. J.* **41**, 261.
Hirs, C. H. W., Moore, S. & Stein, W. H. (1954). *J. Amer. chem. Soc.* **76**, 6063.
Howard, G. A. & Martin, A. J. P. (1950). *Biochem. J.* **46**, 532.
James, A. T. & Martin, A. J. P. (1952). *Biochem. J.* **50**, 679.
Kalbe, H. (1954). *Z. Physiol. Chem.* **297**, 19.
Kleiber, M., Black, A. L., Brown, M. A., Luick, J., Baxter, C. F. & Tolbert, B. M. (1954). *J. biol. Chem.* **210**, 239.
Kumar, S., Lakshmanan, S. & Shaw, J. C. (1959). *J. biol. Chem.* **234**, 754.
Lauryssens, M., Verbeke, R. & Peeters, G. (1957). Proc. Unesco Conf. on Radio-isotopes in Scientific Research, Paris, paper 117.
Lauryssens, M., Verbeke, R., Peeters, G. & Donck, A. (1959). *Biochem. J.* **73**, 71.
McClymont, G. L. (1951). *Aust. J. agric. Res.* **2**, 92.
Peeters, G., Coussens, R. & Sierens, G. (1953). *Arch. int. Pharmacodyn.* **95**, 153.
Peeters, G. & Massart, L. (1947). *Arch. int. Pharmacodyne.* **74**, 83.
Phares, E. F., Mosbach, E. H., Denison, F. W. & Carson, S. F. (1952). *Anal. Chem.* **24**, 660.

POPJÁK, G., FRENCH, T. H., HUNTER, G. D. & MARTIN, A. J. P. (1951). *Biochem. J.* **48**, 612.
REISS, O. K. & BARRY, J. M. (1953). *Biochem. J.* **55**, 783.
SHAW, J. C., LAKSHMANAN, S., CHUNG, A. C., LEFFEL, E. C. & DOETSCH, R. N. (1958). Proc. U.N. Conf. on peaceful uses of atomic energy, Geneva, paper 813.
STEIN, W. H. & MOORE, S. (1948). *J. biol. Chem.* **176**, 537.
TWITCHELL, E. (1921). *J. industr. Engng Chem.* **13**, 806.
VERBEKE, R., AQVIST, S. & PEETERS, G. (1957). *Arch. int. Physiol. Biochim.* **65**, 433.
VERBEKE, R., LAURYSSENS, M., PEETERS, G. & JAMES, A. T. (1959). *Biochem. J.* **73**, 24.
WHISTLER, R. L. & DURSO, F. (1950). *J. Amer. chem. Soc.* **72**, 677.
WOOD, H. G., JOFFE, S., GILLESPIE, R., HANSEN, R. G. & HARDENBROOK, H. (1958). *J. biol. Chem.* **233**, 1264.

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Phage resistance in pure strains of cheese starter streptococci

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SUMMARY. The ratio of phage-resistant to phage-susceptible chains in five strains of *Streptococcus cremoris* and two of *Str. lactis* was investigated. It was shown that this ratio varied between strains and was decreased when the cultures were subcultured with extremely small inocula (< 50 chains/ml) and increased with relatively large inocula (1 %). It is suggested that resistant cells arise by mutations occurring in the absence of phage.

The investigation of the flora of young cheese is handicapped by the preponderance of lactic-acid streptococci derived from the starter. It was suggested (Robertson, 1960) that the starter streptococci be lysed by bacteriophage on plates prepared from cheese emulsion. However, when the non-streptococcal flora of the cheese was very sparse the phage-resistant streptococci, which were thought to have developed from the starter strain used, were so numerous that the non-starter flora could not be examined in spite of the use of bacteriophage. This led to the present examination of the numbers of phage-resistant chains in starter cultures in common use.

Pure cultures of single-strain starter streptococci contain some cells which are not lysed by homologous phage. Recently Prouty & Parisot (1957) measured the numbers of phage-resistant chains in such cultures.

Phage resistance is a hereditable characteristic, for phage-resistant isolates produce pure cultures of resistant cells. Luria & Delbruck (1943) have postulated that such hereditable phage resistance may arise in either of two ways, by mutation or by acquired immunity. By their definition mutation is a change which occurs in bacteria independently of the presence of phage and the proportion of mutants would increase with time as new resistant cells add to the descendants of earlier mutations. Acquired immunity is the ability of a cell to survive an attack by phage and would only be expressed under the action of phage. Where this applies the proportion of resistant cells would depend on such factors as the physiological state of the culture.

The results which follow indicate that phage-resistant cells in cultures of starter streptococci arise by mutation, for the proportion of resistant chains can be influenced by the size of inoculum used.

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MATERIALS AND METHODS

Seven starter strains and their homologous bacteriophages were obtained from the National Collection of Dairy Organisms, Shinfield. Five of these were strains of *Str. cremoris* (ML_4, NCDO 1201; C_{13}, NCDO 1217; E_8, NCDO 1196; HP, NCDO 607; NCDO 804) and two of *Str. lactis* (ML_3, NCDO 763; NCDO 509). With the exception of 804 and 509 all these strains are in regular use in cheese starters in New Zealand.

The starter strains were maintained by daily transfer (1 % inoculum) in autoclaved skim-milk and incubated at 22 °C. The method of determining the numbers of chains containing some phage-resistant cells (resistant chains) in a culture was essentially that described earlier by Robertson (1960). The total counts were determined on glucose—lactose—yeast—phosphate agar (GLYPA) and the phage-resistant counts by absorbing 0·25 ml high-titre phage preparation to 1 ml of a dilution of the culture for at least 10 min before the addition of GLYPA. Counts were made in duplicate and plates were incubated for 3 days at 30 °C. The solid medium GLYPA differed from an earlier modification (Robertson, 1960) of the medium devised by Hunter (1946) in so far as half of the lactose was replaced by glucose.

Cultures from micro-inocula were obtained by serially diluting a 24 h culture (coagulated) in 9 ml portions of milk and incubating the 10^{-9} dilution until coagulated (less than 48 h). The 10^{-10} dilution usually did not clot even on prolonged incubation.

RESULTS AND DISCUSSION

The proportion of phage-resistant organisms in milk cultures of each of seven strains of starter streptococci was determined on several occasions over a period of 8 months. The results are presented in Table 1 under the heading 'normal' and are expressed as the number of resistant chains per million. Although the total counts of the clotted cultures were approximately constant at 7×10^8 chains/ml, the proportion of resistant chains differed markedly from strain to strain, but were relatively constant within each strain. Similar observations were reported by Prouty & Parisot (1957) and a comparable proportion of phage-resistant chains were found in cultures of the one strain (HP) which was common to both determinations.

If the mutation hypothesis applies, a normal inoculum of any of the strains tested is likely to transfer some resistant cells to the daughter culture. By taking a sufficiently small inoculum the chance of a resistant bacterium being transferred may be reduced to insignificance when the proportion in the original culture is low. Any resistant chains detected in the resultant culture will have arisen during growth and would be fewer in numbers than where resistant cells were present in the inoculum. Table 1 shows that with one exception (first determination, strain 804) the proportion of phage-resistant chains in a coagulated culture grown from a micro-inoculum (containing < 50 chains) was lower than in a culture grown from a normal inoculum. With the *Str. lactis* strains the reduction in resistant chains was more pronounced than with *Str. cremoris*.

There was a subsequent increase in the proportion of phage-resistant chains as cultures initially developed from micro-inocula were maintained in daily transfer using a normal, 1 % inoculum (Table 2).

These results show that many of the phage-resistant cells arise as a result of mutation. The present investigation does not rule out the possibility that some of the resistant cells arose as a result of acquired immunity, though this is unlikely for the physiological factors tested by Prouty & Parisot (1957) had no effect on the proportion of resistant chains. Luria & Delbruck (1943) have established that phage resistance in *Escherichia coli* is due to mutation. These workers found that in cultures grown from micro-inocula the variability in numbers of resistant cells was far greater than could be accounted for by experimental error. This variability was shown to be due to the irregular times at which mutation occurred, for this can lead to large differences in the numbers of resistant offspring in a given culture at the time of testing.

Table 1. *Comparison of numbers of phage-resistant chains per million in cultures for which normal* (1 %) *and micro-* (1×10^{-7} %) *inocula were used. The micro-inocula contained* < 50 *chains*

	Streptococcus cremoris										Streptococcus lactis			
	HP		C_{13}		804		E_8		ML_4		ML_3		509	
Type of inoculum...	Normal	Micro	Normal	Micro	Normal	Micro	Normal	Micro	Normal	Micro	Normal	Micro	Normal	Micro
No. of resistant chains per million	1·5	1·2	200	0·9	4·9	10	20	3·1	4·1	2·7	79	0·57	1950	100
	12	2·0	—	2·0	7·0	4·1	86	3·4	4·7	0·47	182	1·30	1530	97
	—	—	—	—	—	—	—	—	2·0	0·27	107	1·20	1510	111
	—	—	—	—	—	—	—	—	2·4	1·08	137	2·2	1590	111
	—	—	—	—	—	—	—	—	7·0	3·9	120	1·54	—	—
	—	—	—	—	—	—	—	—	5·8	—	196	1·48	—	—
	1·6*	—	—	—	—	—	—	—	—	—	52	1·56	—	—
	—	—	—	—	—	—	—	—	—	—	65	8·6	—	—
	—	—	—	—	—	—	—	—	—	—	115	2·8	—	—
Mean	—	—	—	—	—	—	—	—	4·18	1·68	117	2·37	1643	105

* Mean value found by Prouty & Parisot (1957) for HP grown at 20 °C for 24 h.

Table 2. *Increase in numbers of phage-resistant chains per million on daily subculture* (1 %) *of cultures from micro-inocula. The different experiments were made at intervals of some weeks*

No. of transfers since micro-inoculation	ML_4				ML_3				509	
0	2·68	0·47	1·08	3·88	1·30	1·20	1·54	1·48	173	111
5	3·57	1·70	—	16·4	2·63	5·8	—	—	—	—
10	6·1	—	—	—	12·7	—	—	—	342	314
20	7·8	—	5·5	—	—	—	10·0	141	1480	—
40	—	—	—	—	—	—	50·0	—	—	—

Similar techniques were applied to starter streptococci in the present investigation, but the variance was often found to be only slightly larger than the experimental variance. This is probably due to the chain-forming characteristic of streptococci. The mutations will occur in the same irregular way as in single-celled species, and each new mutation will be detectable after it occurs, but there will be no count of the offspring of each mutation while multiplication takes place along a chain. One resistant cell, or a whole chain of resistant cells, will only be expressed once in a plate count. Thus, except when chains are short the build-up of resistant cells will be much faster than is apparent from the increase in resistant count.

Support for this explanation resulted from trials in which cultures were submitted

to vigorous agitation (Ato-mix blender). This treatment reduced chain length, increased total count, slightly increased the phage-resistant count but reduced the proportion of resistant chains, as would be expected if only some of the cells in a chain were resistant. This effect would be expected to be much less pronounced in cultures derived from normal inocula where many chains would be composed entirely of resistant cells.

The observed difference between the numbers of resistant chains in cultures developed from normal and micro-inocula is definite, but less striking than might be expected. For the reasons outlined above, the true difference in the number of resistant cells may be much greater than the difference in resistant count. In the short-chained species (*Str. lactis*) the observed difference is greatest. It is possible that the relatively slow build-up of resistant count may be due, amongst other things, to the following: (1) a generation time for resistant cells appreciably greater than that of susceptible cells, (2) a high rate of mutation back to phage susceptibility, (3) resistant cells arising by adaptation as well as by mutation.

The present investigation was undertaken to determine the suitability of various strains of starter streptococci for making experimental cheese which would contain low numbers of phage-resistant streptococci. Some strains 'naturally' contain very small numbers of phage-resistant chains, and these strains will be the most suitable for making special experimental cheese. It has also been possible to exert some control over the proportion of resistant cells in a culture of any particular strain. The proportion of phage-resistant cells in a cheese starter, and hence in a cheese, may be reduced several-fold by using an inoculum which is sufficiently small to be unlikely to contain resistant cells. For some strains a micro-inoculum containing 10000 chains would be satisfactory. In future experiments where the non-starter flora of a young cheese is to be examined, the starter used for cheese-making will be grown from micro-inocula of strains which naturally contain low numbers of phage-resistant chains.

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REFERENCES

HUNTER, G. J. E. (1946). *J. Dairy Res.* **14**, 283.
LURIA, S. E. & DELBRUCK, M. (1943). *Genetics*, **28**, 491.
PROUTY, C. C. & PARISOT, D. (1957). *J. Dairy Sci.* **40**, 1193.
ROBERTSON, P. S. (1960). *J. Dairy Res.* **27**, 1.

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Studies on the manufacture of sweet cream starter butter

III. Effect of age of starter cultures at time of inoculation

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Summary. The use of starter cultures prepared 3 days, 2 days and 1 day before inoculation at the 1% level into cold cream in the making of sweet cream starter butter was investigated. With *Streptococcus diacetilactis* starter there was little effect of age of starter on diacetyl production in the cream and on the quality of the fresh butter, but when 3-day-old culture was used in the cream there was a slight reduction in the butter quality after 4 and 8 months' storage. With the mixed commercial cultures *Camb* and F.D. increase in age of the culture led to reductions in diacetyl production in the cream. The difference was attributed to the effect of holding for the longer time under acid conditions on the equilibrium between streptococci and leuconostocs in the mixed culture. The age of the *Camb* culture had no effect on the grade of the butter, but holding the F.D. culture for 3 and 2 days instead of for 1 day caused a fall in grade of the butter after storage for 4 and 8 months at 14°F.

At the low temperatures common for the holding of sweet cream for butter-making added starters form diacetyl in considerable proportions without producing significant quantities of lactic acid (Fabricius & Hammer, 1937; Wiley, Cox & Whitehead, 1939; McDowall, Singleton & Le Heron, 1960*a*, *b*). The mechanism of the production of diacetyl and acetoin under these conditions has not been established. It is not known, for example, whether all the diacetyl is formed as a metabolic product of slow growth, or whether it is in part formed by action of enzymes already present in the added culture.

Holding a mixed starter in a fully ripened condition can cause a reduction in its diacetyl and acetoin contents and overlong ripening of cream in the making of acid cream butter affects the flavour of the cream and of the resultant butter. In the making of sweet cream starter butter, there is no information on the effect of over-ripening of starters, before they are added to cold cream, on the diacetyl production of the starter in the cream and on the butter made from it.

EXPERIMENTAL

Three batches of a skim-milk were steam-sterilized. One of these was inoculated with a culture of the starter under investigation 3 days before it was required, a second was inoculated with the same starter 2 days before it was required, and the

third was inoculated the day before it was required as is usual in the making of sweet cream starter butter. The inoculations were from mother cultures subcultured daily. Comparisons were made with three starters, namely, the single strain of *Str. diacetilactis* and the mixed commercial starters *Camb* and F.D. used in the previous investigations (McDowall *et al.* 1960*a*, *b*).

The cultures were added to batches of the same Vacreator-treated cream, which was then held in cream-holding vats at the normal holding temperatures for the making of sweet cream butter. Butters were made, creams and butters were analysed, and the butters were stored and graded, as described in Parts I and II of this series (McDowall *et al.* 1960*a*, *b*).

RESULTS

Effect on acidity of cream at churning. The results in Table 1 show that holding the culture for 2 days instead of 1 resulted in a slight increase in the acidity of the starter itself. At the holding temperatures of the cream, 40–46 °F, there was no production of acid by any of the starters in the cream between the time of inoculation and the time of churning (20 h). These results are in line with those of the earlier investigation on the effect of holding temperature of cream inoculated with 1-day-old cultures (McDowall *et al.* 1960*a*).

Diacetyl and acetoin in starter. The results in Table 2 show that ageing had a variable

Table 1. *Effect of age of culture added to cold cream on the diacetyl and acetoin contents of the cream at churning and of the fresh butter*

Serial no., and age of culture in days	Starter used	Acidity, % lactic acid				Temperature, °F		pH of the fresh butter
		Starter	Cream off cooler	Cream after adding starter	Cream at churning	Holding*	Churning	
I–1	*Str. diacetilactis* 1%	0·80	0·06	0·07	0·07	42, 40·5 (9)	43·5	6·74
2		0·88	0·06	0·07	0·075	42, 40·5 (9)	43·5	6·74
3		0·88	0·06	0·07	0·07	42, 41 (9)	43·5	6·73
II–1	*Str. diacetilactis*, 2%	0·82	0·06	0·07	0·075	44, 40·5 (7) 45 (20)	46	6·58
2		0·92	0·06	0·07	0·075	44, 40 (7) 44·5 (22)	46	6·41
3		0·92	0·06	0·075	0·075	44, 39·5 (7) 43·5 (24)	46	6·66
III–1	*Camb*, 1%	0·93	0·07	0·08	0·08	44, 42·5 (9) 42 (20)	45·5	6·86
2		0·99	0·07	0·075	0·075	44, 40·5 (9) 44 (22)	45·5	6·86
3		0·94	0·07	0·075	0·075	44, 40·5 (9) 46 (20)	45	6·86
IV–1	F.D., 2%	0·81	0·065	0·08	0·08	45, 43·5 (9) 45 (20)	50	6·56
2		0·91	0·065	0·08	0·08	48, 41·5 (9) 45·5 (20)	50	6·56
3		0·95	0·065	0·08	0·08	48, 42·5 (9) 45·5 (20)	50	6·58

* The holding times in hours are shown in brackets.

effect on the diacetyl content of a *Str. diacetilactis* culture. In trial I the diacetyl content was highest when the culture was 2 days old, and in trial II it was highest when the culture was 1 day old. Ageing of the culture of the mixed starter *Camb* caused a small but definite reduction in the diacetyl content of the starter, and corresponding ageing of the culture of the mixed starter F.D. caused a very marked reduction.

There were no appreciable changes in the acetoin content of *Str. diacetilactis* cultures with age, but very marked reductions for the cultures of the mixed starters *Camb* and F.D.

Table 2. *Effect of age of culture added to cold cream on the diacetyl and acetoin contents of the cream at churning and of the fresh butter*

Serial no., and age of culture in days	Starter used	Diacetyl, ppm.			Acetoin, ppm.			Diacetyl/acetoin ratio	
		Starter	Cream	Butter	Starter	Cream	Butter	Cream at churning	Butter
I–1	*Str. diacetilactis*, 1%	0·82	0·34	0·075	214	5·80	0·86	0·059	0·087
2		1·32	0·34	0·07	209	5·80	0·74	0·059	0·085
3		1·22	0·38	0·075	206	3·80	0·66	0·100	0·114
II–1	*Str. diacetilactis*, 2%	1·68	1·00	0·16	194	13·6	2·32	0·074	0·069
2		0·66	1·12	0·15	183	12·8	2·12	0·087	0·071
3		0·76	0·90	0·14	177	9·2	1·64	0·098	0·085
III–1	*Camb*, 1%	0·46	0·19	0·045	150	4·20	0·76	0·045	0·059
2		0·40	0·09	0·03	77	2·30	0·56	0·039	0·054
3		0·34	0·10	0·025	12	1·20	0·46	0·083	0·053
IV–1	F.D., 2%	1·28	0·32	0·05	111	4·80	0·92	0·067	0·083
2		0·16	0·23	0·045	8	2·30	0·48	0·100	0·094
3		0·14	0·15	0·035	6	1·70	0·70	0·088	0·050

Diacetyl and acetoin in cream at churning. Production of diacetyl by *Str. diacetilactis* in the cold cream (Table 2) was not greatly affected by the age of the cultures—the differences were not greater than could have been caused by unavoidable variations in the holding temperature of the cream in the storage vats. Increase in the age of the *Str. diacetilactis* cultures from 2 to 3 days caused a definite reduction in acetoin formation. With the mixed cultures age of starter caused a definite reduction in both diacetyl and acetoin formation in the cold cream. There was a rise in the diacetyl/acetoin ratio in the cream at churning with increase in the age of the culture.

Diacetyl and acetoin in butter during storage. It is evident from Table 3 that age of starter, over the range 1–3 days, had no effect on the rate of change in diacetyl and acetoin contents of the butter during storage at 14 °F for 4 and 8 months. There was, as in the previous investigations (McDowall *et al.* 1960*a*, *b*) a slow but steady loss of diacetyl and, particularly with *Str. diacetilactis*, a tendency for the acetoin content to rise during storage.

Gradings of fresh and stored butters and oxidation tests. The results in Table 4 show that the age of the starter at the time of inoculation into the cold cream had no effect on the grade of the fresh butter, and with *Str. diacetilactis* and *Camb* starters it had only a slight effect on the grade scores of the butters after 4 and 8 months' storage at 14 °F. With F.D. starter, on the other hand, there was an appreciable loss

in the quality of the butter during storage, and the loss was greater for the butters made from cream treated with the 2-day-old and 3-day-old cultures. The down-grading was due to the presence of an 'oily' flavour.

There was no effect of age of culture at time of inoculation on the oxidation test results and on the acidity values for butterfats extracted from the butters after 4 and 8 months' storage. This applied also to the butters in trial IV which were down-graded for 'oily' flavour.

Table 3. *Effect of age of culture added to cold cream on the diacetyl and acetoin contents of the butters when fresh and after* 4 *and* 8 *months' storage at* 14 °F

Serial no. and age of culture in days	Starter used	Diacetyl in butter, ppm.			Acetoin in butter, ppm.		
		Fresh	4 months	8 months	Fresh	4 months	8 months
I–1	*Str. dia-*	0·075	0·06	0·05	0·86	0·94	0·98
2	*cetilactis*, 1 %	0·07	0·055	0·06	0·74	0·80	0·90
3		0·075	0·055	0·05	0·66	0·82	0·72
II–1	*Str. dia-*	0·16	0·15	0·13	2·32	2·16	2·40
2	*cetilactis*, 2 %	0·15	0·17	0·14	2·12	2·12	2·45
3		0·14	0·15	0·13	1·64	1·76	1·80
III–1	*Camb*, 1 %	0·045	0·035	0·04	0·76	0·80	0·70
2		0·03	0·025	0·02	0·56	0·72	0·56
3		0·025	0·03	0·025	0·46	0·52	0·58
IV–1	F.D. 2 %	0·05	0·05	0·055	0·92	0·96	0·82
2		0·045	0·045	0·035	0·48	0·54	0·62
3		0·035	0·04	0·03	0·70	0·64	0·60

Table 4. *Effect of age of culture added to cold cream on the gradings of butter when fresh and after* 4 *and* 8 *months' storage at* 14 °F

Serial no., and age of culture in days	Starter used	Grade score of butter		
		Fresh	after 4 months	after 8 months
I–1	*Str. diacetilactis*, 1 %	93½	93½	93½
2		93½	93½	93½
3		93½	93	93
II–1	*Str. diacetilactis*, 2 %	93½	93	93
2		93	93	92½
3		93	92½	92
III–1	*Camb*, 1 %	93½	93½	93½
2		93½	93	93½
3		93½	93	93½
IV–1	F.D., 2 %	93½	92½	92
2		93½	91	90½
3		93½	91	90½

DISCUSSION

Absence of any marked effect of age of *Str. diacetilactis* culture inoculated into cold cream on the diacetyl production during the holding period indicated that there was no radical change in the condition of the culture while it remained comparatively dormant at the pH of the ripe culture.

The marked changes in capacity to produce diacetyl which occurred with the mixed cultures were probably the result of changes in the relative proportions of the different organisms present. As shown by Wiley *et al.* (1939), diacetyl is produced in the cold sweet cream by streptococci and not by the leuconostocs. The acid conditions present in a ripe starter are known to be favourable for leuconostocs, and growth of these organisms could be expected to suppress the streptococci, which are inhibited by high hydrogen ion concentrations. The fall in diacetyl production with increase in period of ripening of the mixed starter was thus probably a result of an increasing predominance of the leuconostocs over the streptococci in the starter culture at the time of inoculation into the cold cream.

Irrespective of the effect on its capacity to produce diacetyl, a starter as it becomes over-ripe can undergo changes in character as indicated by organoleptic examination. For this reason manufacturers of sweet cream starter butter usually prefer to avoid the use of over-ripe starter cultures. The grading results of the fresh butters (Table 4) show that with *Str. diacetilactis* starter an increase in age of the culture at time of inoculation did not result in the appearance of any objectionable flavour in the fresh butter, although it led to a slight down-grading of the stored butters. With the mixed cultures there was not sufficient carry-over of objectionable flavour from the older cultures to cause a down-grading of the fresh butter. With starter *Camb* there were no differences in grade even after 4 and 8 months' storage of the butter, but with starter F.D. use of an over-ripe culture caused a down-grading of the butter after storage.

CONCLUSIONS

Since with all the starters investigated the capacity to produce diacetyl in the cold cream did not increase when the culture was held for more than 1 day, there is no incentive for the use of over-ripe cultures. Where, however, it is more convenient in practice to prepare starters at less frequent intervals than daily, the indications of the above results are that *Str. diacetilactis* cultures can safely be grown for at least 2 days at 70°F before they are inoculated into cold cream, without serious loss of capacity to produce diacetyl during the holding period and without significant loss of quality of the resultant butter either fresh or after storage for 4 and 8 months at 14°F. This does not apply to the mixed starters investigated.

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REFERENCES

Fabricius, N. E. & Hammer, B. W. (1937). *Res. Bull. Ia agric. Exp. Sta.* no. 221.
McDowall, F. H., Singleton, J. A. & Le Heron, B. S. (1960*a*). *J. Dairy Res.* **27** 81.
McDowall, F. H., Singleton, J. A. & Le Heron, B. S. (1960*b*). *J. Dairy Res.* **27**, 91.
Wiley, W. J., Cox, G. A. & Whitehead, H. R. (1939). *J. Counc. sci. industr Res. Aust.* **12**, 232.

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The use of ultrafiltration and dialysis in isolating the aqueous phase of milk and in determining the partition of milk constituents between the aqueous and disperse phases

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Summary. A description is given of various methods for the ultrafiltration and dialysis of milk and of the composition of the sera obtained. Ultrafiltrate prepared by the procedure recommended is reasonably representative of the aqueous phase of milk, but its content of lactose and citric acid, and consequently also of calcium, is determined to a slight degree by the sieving phenomenon known to occur often in ultrafiltration. The composition of diffusate obtained from milk at 20 °C is not thought to be controlled to any significant extent by a Donnan effect and is regarded as identical with that of the aqueous phase of milk. The lactose content of diffusate suggests that about 2 % of the water in milk is bound to protein, and allowance should be made for this when calculating the concentrations of the soluble constituents in milk from the composition of diffusate. Diffusate prepared from milk at 3 °C contains slightly more total calcium, ionized calcium and phosphorus than diffusate prepared at 20 °C. These differences are attributed to a change in the partition of calcium and phosphorus between the disperse and aqueous phases at the lower temperature, an explanation that is supported by the reversibility of the change. The composition of diffusate prepared by the procedure recommended indicates that about 5 % of the sodium and about 6 % of the potassium and citric acid in milk are in the disperse phase.

It is sometimes desirable to know not only the total concentration in milk of a given constituent but also the amounts of it in the disperse and aqueous phases, i.e. how much can be regarded as 'colloidal' and how much as 'soluble'. This information is usually obtained by determining the total amount of the constituent and the amount in solution; the colloidal fraction is then obtained by difference. Such a fractionation depends on the preparation of a suitable serum for estimating the soluble constituents. This serum should have the same composition as the aqueous phase of milk and therefore in its preparation the equilibria in milk should be disturbed as little as possible. Several methods of preparing a serum are available and of these the most suitable would appear to be ultrafiltration or dialysis. Other methods of estimating the soluble constituents of milk are the analysis of rennet whey or the serum obtained by high-speed centrifuging, and the treatment of milk with ion-exchange resins.

In an earlier investigation by the present authors (White & Davies, 1958), the partition of calcium, magnesium, citric acid and phosphorus between the disperse and aqueous phases of separated milk was achieved by filtering the milk, at 20 °C, through Cellophane at a pressure of 15 lb/in² in a modification of the apparatus devised by Clark (1951). Subsequently, a comparison was made of the values for the soluble fraction of these constituents obtained by analysing ultrafiltrates prepared as above and diffusates prepared at 3 °C by a static dialysis method (Lampitt & Bushill, 1933; Pyne, 1940). The average results from ten different samples of separated milk (Table 1) show that the dialysis procedure gave higher values for soluble calcium, citric acid and phosphorus; the percentage difference between the two values for each constituent showed little variation in the ten milks. As the differences between the values for soluble calcium, citric acid and phosphorus obtained by ultrafiltration and dialysis exceeded any that could be atttributed to analytical error, it was decided to examine the composition of ultrafiltrate and diffusate prepared under various conditions, and also that of rennet whey and centrifuge serum, to decide which serum most closely represented the aqueous phase of milk.

Table 1. *Average concentration of soluble calcium, magnesium, citric acid and phosphorus in ten samples of separated milk determined by ultrafiltration at* 20 °C *and dialysis at* 3 °C

	Ultrafiltration (*a*)	Dialysis (*b*)	$\frac{(b)}{(a)} \times 100$
	mg/100 g milk		
Calcium	36·1	39·9	110·5
Magnesium	7·8	7·8	100·0
Citric acid	160	168	105·0
Phosphorus	38·0	39·7	104·5

EXPERIMENTAL

Ultrafiltrate

Preparation of sera

With Cellophane sheet. In this method, Cellophane (P.T.300; British Cellophane Ltd), soaked in a large volume of distilled water overnight and then superficially dried with filter paper, is used as the filtering membrane in an improved model of the apparatus described by Clark (1951). The apparatus (Fig. 1) consists of five neoprene rubber gaskets (15 × 15 cm; 2 cm wide; 0·3 cm thick), separated by four sheets of Cellophane (15 × 15 cm), clamped between end-plates (chromium-plated brass or stainless steel; 15 × 15 × 0·6 cm) lined with Alkathene (polythene) film (15 × 15 cm; gauge 500; British Visqueen Ltd). Each of the rubber gaskets has a small hole (0·4 cm dia.) in one corner and in three of them this hole is connected to the interior of the gasket with a piece of stainless-steel hypodermic needle (0·1 cm dia.); the spaces enclosed by these three gaskets serve as milk-holding chambers. The other two gaskets have a piece of needle passing through the corner diagonally opposite the hole to connect the interior of the gasket to the exterior, and each contains a piece of Perspex (11 × 11 × 0·2 cm) between two pieces of rayon-base filter paper (P.375.F; Postlip Paper Sales Ltd.) cut to a shape so that they expand to 11 × 11 cm when

wetted, and placed so that the unidirectional fibres are parallel to the diagonal joining the hole and the outlet tube of the gasket. The spaces enclosed by these two gaskets act as collecting chambers for the ultrafiltrate; the Perspex and paper support the Cellophane and promote drainage of the ultrafiltrate through the outlet tubes. After the apparatus is assembled, with the three milk-holding gaskets occupying the middle and the two end positions, a hole is pierced in one corner of each sheet of Cellophane by passing a wire through the inlet tube on one of the end-plates and the hole in the corner of each gasket. In the apparatus described, the filtering area of the Cellophane is $11 \times 11 \times 4 = 484$ cm^2.

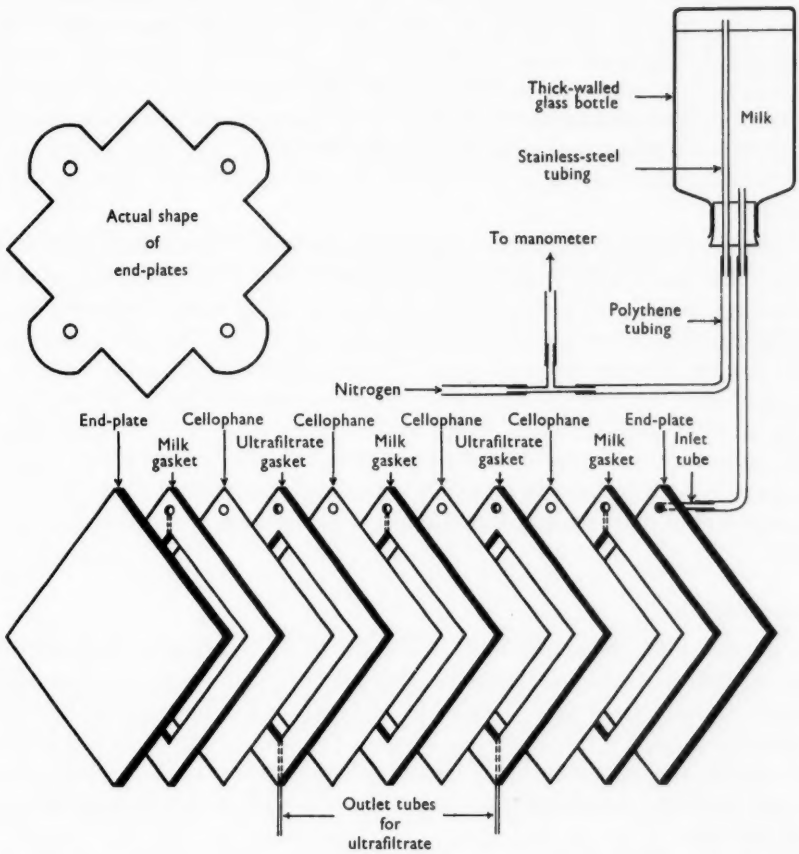

Fig. 1. Diagrammatic arrangement of ultrafiltration apparatus (after Clark, 1951)

In the preparation of ultrafiltrate with this apparatus, 400 ml of separated milk are placed in a thick-walled glass bottle (a blood transfusion bottle is suitable) which is connected by transparent polythene tubing to the inlet tube of the apparatus and to a mercury manometer and a cylinder of compressed nitrogen. The inverted bottle is supported above the apparatus so that when pressure is alternately applied and released, the air in the milk-holding chambers escapes and they become filled with

milk (approx. 140 ml). A steady pressure, usually 38 cm of mercury, is then applied and the ultrafiltrate collected at the two outlet tubes at the bottom of the apparatus.

With Visking tubing. In this method, washed and dried Visking dialysis tubing (1·4 cm inflated diameter; Hudes Merchandising Corporation Ltd.) is used as the filtering membrane. A piece of the tubing, knotted and tied over a small flat-bottomed sample tube at one end and tied over a piece of glass tubing at the other, is inserted in a stainless-steel tube (93 cm long; 1·9 cm internal dia.) which is lined with Alkathene film (gauge 500–1000) and two thicknesses of rayon-base filter paper with the fibres running lengthways along the tube. The bottom of the stainless-steel tube is closed with a rubber bung fitted with a stainless-steel outlet tube and the annular space between the top of the steel tube and the glass tube is sealed with a rubber sleeve. The glass tube is connected to a glass bottle containing 400 ml of separated milk, which is supported in an inverted position above the steel tube. Pressure is then applied as described above. The Visking tube holds about 160 ml of milk and has a filtering area of approximately 400 cm^2.

Diffusate

To prepare diffusate, Visking tubing as described above is used. A piece of this tubing, about 65 cm long, is tied at one end and a sinker (glass stopper) attached; the other end is tied over a piece of glass tubing. Distilled water is pipetted into the tube which is then twisted and tied just above the water level. The tube is immersed in a large volume of separated milk (in a cylinder) which is maintained at 20 or 3 °C until equilibrium between the water and the aqueous phase of the milk is established. The top of the cylinder is covered with Alkathene film during the equilibration period. In dialysis at 20 °C, 0·25 ml of chloroform per 100 ml of milk is added as a preservative.

Variations in the techniques of ultrafiltration and dialysis and their effect on the composition of ultrafiltrate and diffusate will be described in the section dealing with results.

Rennet whey

To prepare whey, separated milk is warmed to 30 °C, 0·5 ml of dialysed rennet added per 100 ml of milk and the mixture kept at 30 °C until a firm curd forms (about 15 min). The coagulated milk is cooled to 20 °C, left at this temperature for 1 h, and then filtered through a Whatman No. 1 paper. The whey obtained by this process is unsuitable for the determination of ionized calcium because of its turbidity (mainly from residual fat). Accordingly, a portion of whey is clarified by centrifuging it for 3 h at 50000*g*.

Centrifuge serum

'Centrifuge serum' is obtained by centrifuging separated milk for 3 h at 50000*g*; the temperature of the milk should be maintained at approximately 20 °C during the centrifuging. The traces of fat floating on the supernatant serum are removed by suction. The serum is then decanted and filtered through a Whatman No. 1 paper. Serum so prepared has only a very slight turbidity.

Milk samples

Bulk milk, herd bulk milk and milk from individual cows were used. The bulk samples were obtained at a local creamery from a tank holding about 2000 gal of separated milk; the temperature of these samples, which was about 10 °C on arrival at the laboratory, was raised to 20 °C. The herd bulk and individual cow milk samples were collected from the Hannah Institute herd of Ayrshire cows. These samples were warmed to 30 °C and the fat removed in an electrically driven separator, or by centrifuging for 30 min at 1000*g*, and the separated milk immediately cooled to 20 °C. All the separated milks contained about 0·1 % of fat. The analytical values in this paper therefore apply to separated milk and not to whole milk.

Analytical methods

The milk samples were analysed for the following constituents: total solids, total nitrogen, non-protein nitrogen (N.P.N.), lactose, calcium, magnesium, sodium, potassium, phosphorus, citric acid and chloride. The sera were analysed for total solids, total nitrogen, lactose, total calcium, ionized calcium, magnesium, sodium, potassium, total phosphorus, inorganic phosphorus, citric acid and chloride. The pH of the milks and sera was also determined. The analytical methods used were the same as those previously outlined (White & Davies, 1958) except that sodium and potassium were determined with a flame photometer (Evans Electroselenium Ltd.) by the method of van der Have & Mulder (1957). In determining the lactose content of the sera, the sera were treated with the NaOH-$ZnSO_4$ protein precipitant used with the milk samples and the filtrates analysed.

Table 2. *Reproducibility of the analytical procedure as shown by the analysis of five portions of an ultrafiltrate from separated milk*

	Portion					Maximum variation
	1	2	3	4	5	
	mg/100 g milk					
Total calcium	39·8	39·7	39·8	39·8	39·7	0·1
Ionized calcium	11·1	11·3	11·5	11·5	11·4	0·4
Magnesium	8·5	8·2	8·2	8·2	8·3	0·3
Sodium	42	42	42	42	41	1
Potassium	139	140	141	141	140	2
Total phosphorus	43·1	43·6	43·6	43·6	44·1	1·0
Inorganic phosphorus	34·4	34·6	34·4	34·4	34·2	0·4
Citric acid	170	172	173	169	172	4
Chloride	93·2	93·2	93·4	93·8	94·0	0·8
Total nitrogen	26·7	26·7	26·7	26·8	27·0	0·3
Lactose (anhydrous)	4840	4840	4830	4840	4850	20

All the analyses were done in duplicate. Where filtrates were used, correction was made for the volume of the precipitate. The concentrations of the constituents in the various sera were determined as mg/100 g of serum and converted to mg/100 g of milk by multiplying by the factor (wt. of water in 100 g milk)/(wt. of water in 100 g serum) for ready comparison with the total concentrations in the milk. This method of expressing the analytical results has been used throughout the paper. With diffusate,

allowance was made for the concomitant dilution of the aqueous phase of the milk and for the volume of any preservative added. The extent to which any differences in composition between two sera could be attributed to analytical error was determined by analysing in duplicate five portions of a milk ultrafiltrate (Table 2). The last column in this table indicates the amounts which any differences in composition between two sera should exceed to be considered beyond the limits of experimental error.

RESULTS AND DISCUSSION

The effect of variations in the method of ultrafiltration on the composition of ultrafiltrate

Composition of serial fractions of ultrafiltrate

To determine whether the composition of ultrafiltrate varied during the ultrafiltration process serial 10 ml fractions were obtained from samples of herd bulk milk using Clark's apparatus, Cellophane (P.T. 300) and a pressure of 76 cm of mercury. The composition of ten such fractions is shown in Table 3; three milks similar in

Table 3. *Composition of serial 10 ml fractions of ultrafiltrate from separated milk*

	Fraction										Total in milk
	1	2	3	4	5	6	7	8	9	10	
	mg/100 g milk										
Total calcium	34·6	34·4	34·6	34·6	35·1	35·0	34·8	34·8	35·1	35·1	111·3
Ionized calcium	10·3	9·8	9·9	10·0	10·0	9·9	9·8	10·4	9·9	9·9	—
Magnesium	7·8	8·1	8·1	8·0	7·9	7·9	7·9	7·9	8·1	8·0	12·0
Sodium	43	43	44	43	43	43	43	43	44	43	48
Potassium	130	140	141	141	142	141	141	141	142	141	154
Total phosphorus	46·2	46·4	46·1	46·3	46·1	46·9	46·8	46·4	46·2	47·0	96·9
Inorganic phosphorus	39·5	40·5	39·5	40·4	39·7	39·5	40·1	40·2	40·0	39·6	—
Citric acid	168	165	166	170	170	169	171	169	171	172	180
Chloride	99·8	100·7	101·0	100·8	101·0	101·2	100·9	101·0	100·7	101·0	97·5
Total nitrogen	17·7	17·5	18·1	18·5	18·1	19·0	19·6	19·2	19·6	19·7	17·6*
Lactose (anhydrous, g/100 g milk)	4·67	4·73	4·78	4·80	4·79	4·81	4·82	4·85	4·85	4·87	4·83
pH	6·75	6·76	6·76	6·76	6·77	6·76	6·76	6·76	6·76	6·76	6·70

* Non-protein nitrogen, not total nitrogen

composition were used in compiling this table as all the analyses could not be done on 10 ml of ultrafiltrate. The results show that the first 10 ml fraction contained slightly more ionized calcium and less potassium than the succeeding fractions, in which there were small progressive increases in the concentrations of total nitrogen and lactose and possibly in total calcium and citric acid. Although some of these differences may appear to be within the experimental error (see Table 2), similar trends were found in serial fractions of ultrafiltrate from milks differing considerably in composition from the herd bulk milk. Comparison of the values in the last column of Table 3 with the ultrafiltrate values shows that there was incomplete passage of sodium, potassium and citric acid through the Cellophane, that all the ultrafiltrate fractions indicated a greater concentration of chloride in the milk than that directly

determined and that as ultrafiltration progressed, the fractions became enriched with nitrogen and lactose. There was naturally incomplete filtration of calcium, magnesium and phosphorus since a proportion of these constituents is known to be associated with the casein. The pH of each fraction of ultrafiltrate was slightly greater than the pH of the milk.

It was expected that the composition of the first 10 ml fraction of ultrafiltrate would differ from that of the succeeding fractions because of the residual water content and primary adsorbing capacity of the Cellophane membranes. However, although the total residual water content of the four sheets of Cellophane was found to be about 3·2 ml, there was little evidence of any dilution of the first 10 ml fraction; most of the residual water may be retained permanently by the Cellophane and the remainder, on mixing with the much larger volume of ultrafiltrate required to saturate the rayon-base papers in the ultrafiltrate-collecting chambers, would have a negligible diluting effect.

The primary adsorbing capacity (and permeability) of the Cellophane was examined by the ultrafiltration of milk ultrafiltrate and standard solutions. These experiments showed that sodium, calcium and chloride ions were not adsorbed or retained by the Cellophane, that there was some adsorption of the potassium ion and that a proportion of the relatively large molecules of citric acid (whether as $H_3.Cit$, Cit^{3-} or $CaCit^{-}$) and lactose, and part of the nitrogen, were retained in accordance with the principle of the sieving phenomenon (Ferry, 1936). The operation of the sieving effect can be seen in Table 3 where the concentrations of lactose and total nitrogen, and probably also citric acid, in the serial fractions of ultrafiltrate gradually increase as a result of the progressive rise in the concentrations of these constituents in the unfiltered milk. The experiments showed also that the adsorption of potassium occurred mainly during the collection of the first 10 ml of ultrafiltrate, and that the degree of retention of the potassium ion by sieving was small. This means that the deficiency of potassium in the second to the tenth fractions of ultrafiltrate (Table 3) cannot be attributed to primary adsorption or retention by the Cellophane; a Donnan effect and the presence of protein-bound potassium in milk may be causes of this deficiency. These two factors may also account for the deficiency of sodium in all the fractions. The higher chloride content and the slightly higher pH of all the ultrafiltrate fractions compared with the milk may be other indications of a Donnan effect.

In another experiment, serial 20, 50, 130 and 50 ml fractions of milk ultrafiltrate were collected and the two 50 ml fractions analysed. These fractions were similar in composition, except for the expected higher concentration of nitrogen and lactose in the second 50 ml fraction. This similarity suggested either that any influence of a Donnan effect on the composition of the ultrafiltrate had remained constant despite the fact that the concentration of protein in the milk had approximately doubled by the time the last fraction was collected or, what is more likely, that the composition of the milk ultrafiltrate was not controlled to any significant degree by a Donnan effect. The results of this experiment, taken with those of the foregoing, indicated that the 50 ml of ultrafiltrate collected after the passage of the first 20 ml would be a representative sample. Accordingly, in all succeeding ultrafiltration experiments, the first 20 ml of ultrafiltrate were discarded and the following 50 ml collected for

analysis. This system of sampling is the same as in the earlier work (White & Davies, 1958) and it is also used when preparing ultrafiltrate with Visking tubing.

Reproducibility of the methods of preparing ultrafiltrate

Ultrafiltrates prepared from samples of the same milk under standard conditions, i.e. same membrane, pressure and temperature, were invariably identical in composition. Also, three ultrafiltrates prepared from the same milk under standard conditions, but using Cellophane (P.T. 300) from three different production batches, were very similar in composition. That this result was obtained although one batch of Cellophane was almost 3 years old and had a porosity about half that of the other two new batches (as judged by the respective filtration rates), is evidence that the composition of ultrafiltrate will not vary appreciably with the sample of Cellophane used. However, the ultrafiltrates obtained with the two new batches of Cellophane did contain slightly more lactose and nitrogen as a result of their greater porosity. The decrease in the porosity of Cellophane with age, which results from a decrease in the amount by which it swells when wetted, has been observed also by McBain & Stuewer (1936).

Effect of using different pressures on the composition of ultrafiltrate

Ultrafiltrates were prepared from samples of bulk milk and milk from individual cows using Cellophane (P.T. 300) and pressures of 19, 38 and 76 cm of mercury (Table 4). Apart from the finding that slightly less of the larger molecules, i.e.

Table 4. *Effect of using different pressures on the composition of ultrafiltrate from separated milk (average results from four milks)*

Pressure, cm mercury ...	19	38	76
Rate, ml/h ...	5·3	10·0	18·2
	mg/100 g milk		
Total calcium	33·2	33·0	32·2
Ionized calcium	11·3	11·0	11·1
Magnesium	7·8	7·7	7·7
Sodium	56	57	56
Potassium	137	138	137
Total phosphorus	41·0	40·8	40·6
Inorganic phosphorus	31·9	32·0	31·8
Citric acid	139	138	135
Chloride	119·9	119·9	120·5
Total nitrogen	28·2	28·0	28·2
Lactose (anhydrous)	4440	4440	4370
pH	6·91	6·92	6·92

lactose and citric acid, passed through the Cellophane at the highest pressure, the composition of ultrafiltrate varied little with pressure. As part of the soluble citrate in milk is in the form of calcium citrate, the ultrafiltrate obtained with the highest pressure contained slightly less total calcium than the other two ultrafiltrates. The composition of ultrafiltrate prepared with Visking tubing varied with pressure in exactly the same way.

The rate of filtration was roughly proportional to the pressure but with increasing pressure increasing blockage of the pores of the Cellophane was evident (Table 4).

To minimize any sieving or blocking effects it is desirable to use as low a pressure as possible, but as there was little difference in the composition of the ultrafiltrates obtained at pressures of 19 and 38 cm of mercury, no advantage was to be gained by using a pressure lower than 38 cm. This pressure was consequently adopted as the standard for ultrafiltration. In the earlier work (White & Davies, 1958), a pressure of 15 lb/in^2 (approx. 78 cm mercury) was used and therefore the values now obtained by ultrafiltration for soluble calcium and citric acid are slightly higher.

Effect of using different membranes on the composition of ultrafiltrate

Previous work had shown that of the three non-waterproofed grades of Cellophane sheet currently available, the grade P.T. 300 was the most suitable for the ultrafiltration of milk. Consequently, only this grade of Cellophane, and Visking tubing, were compared (Table 5). The results show that Visking tubing is slightly more permeable than Cellophane to lactose and citric acid and as a consequence of the latter, the Visking ultrafiltrate contained a little more total calcium. These differences were evident whether the ultrafiltration pressure was 19, 38 or 76 cm of mercury. The greater permeability of the Visking tubing to the larger molecules can be attributed to its porosity being about 1·3 times that of the Cellophane, as calculated from the respective filtration rates at each pressure. However, the fact that the concentrations of lactose and citric acid in Visking ultrafiltrate varied with pressure in the same way as they did in Cellophane ultrafiltrate (see previous section) despite the greater porosity of the Visking tubing, was evidence that the sieving effect operated also with this membrane.

Table 5. *Effect of using different membranes on the composition of ultrafiltrate from separated milk (average results from three milks)*

	Membrane	
	Cellophane sheet	Visking tubing
	mg/100 g milk	
Total calcium	33·8	34·3
Ionized calcium	10·9	11·2
Magnesium	7·4	7·4
Sodium	48	51
Potassium	135	136
Total phosphorus	38·6	39·1
Inorganic phosphorus	30·6	31·1
Citric acid	147	155
Chloride	106·8	108·3
Total nitrogen	28·5	28·1
Lactose (anhydrous)	4540	4580
pH	6·93	6·93

Effect of temperature on the composition of milk ultrafiltrate

A possible explanation of the differences in the values for the concentrations of some soluble constituents of milk obtained by ultrafiltration at 20 °C and dialysis at 3 °C (Table 1), is that the partition of these constituents between the aqueous

and disperse phases varies with temperature. To test this hypothesis, ultrafiltrates were prepared at 20 °C from fresh milk, and at 3 °C after the milk had been stored for 24, 48 and 72 h at 3 °C. Cellophane membranes were used. Preliminary experiments showed that when ultrafiltration is conducted under the same pressure at 3 and 20 °C, the rate of filtration at 3 °C is approximately half that at 20 °C, mainly because of the higher viscosity of the milk at the lower temperature. To make temperature the only significant variable during ultrafiltration at 3 and 20 °C, the rate of filtration at each temperature was made equal by using, at 3 °C, a pressure approximately double that used at 20 °C. To verify that bacterial action did not alter the chemical composition of the stored milks, fresh and stored samples were analysed for citric acid, lactose, total acid-soluble phosphorus and inorganic acid-soluble phosphorus, and their pH and nitrogen partition determined. The only changes detected were very small increases in the concentrations of inorganic acid-soluble phosphorus and proteose-peptone nitrogen.

Table 6. *Effect of temperature on the composition of ultrafiltrate as shown by the ultrafiltration at* 20 °C *of fresh separated milk, and at* 3 °C *of samples of the milk after storage for* 24, 48 *and* 72 *h at* 3 °C (*average results from two milks*)

	Sample			
	Fresh (20 °C)	24 h at 3 °C	48 h at 3 °C	72 h at 3 °C
	mg/100 g milk			
Total calcium	35·0	37·4	37·3	37·2
Ionized calcium	11·2	12·6	12·8	12·7
Magnesium	8·0	7·6	7·6	7·6
Sodium	50	50	50	50
Potassium	134	134	135	134
Total phosphorus	39·6	40·5	40·0	40·3
Inorganic phosphorus	31·4	32·8	32·8	32·6
Citric acid	154	150	151	150
Chloride	113·2	114·3	112·4	113·1
Total nitrogen	31·6	30·6	30·4	31·7
Lactose (anhydrous)	4550	4490	4490	4450
pH	6·84	6·91	6·93	6·91

The results in Table 6 show that all the ultrafiltrates obtained from milk stored at 3 °C contained more total calcium, ionized calcium and phosphorus and slightly less citric acid and lactose than the ultrafiltrate obtained at 20 °C from fresh milk. The increase in the concentration of soluble calcium and the probable increase in the concentration of soluble phosphorus suggest that at 3 °C part of the calcium phosphate in the disperse phase at 20 °C had gone into solution. This interpretation is supported by the fact that the pH values of the ultrafiltrates from the milks stored at 3 °C were higher than those of the ultrafiltrates obtained at 20 °C; such a change in pH would be expected if some colloidal calcium phosphate dissolved.

The discrepancy between the difference in the total phosphorus contents of the ultrafiltrates obtained at 3 and 20 °C and the difference in the corresponding inorganic phosphorus values suggests that some hydrolysis of ester phosphorus compounds occurred during storage at 3 °C, and that therefore a more reliable estimate of the temperature-associated changes in phosphorus partition is given by comparing the

total phosphorus values. It would appear from the observations of McBain & Stuewer (1936) that the porosity of Cellophane at 3 °C may be greater than at 20 °C. However, it is very unlikely that any increase in porosity could be responsible for any of the differences in composition of the ultrafiltrates obtained at these temperatures; at 20 °C Cellophane is already permeable to calcium ions. The differences in the concentrations of citric acid and lactose in the ultrafiltrates prepared at 20 and 3 °C may be a pressure-viscosity effect.

If it is correct to attribute the above differences in the composition of ultrafiltrate obtained at 20 and 3 °C to the influence of temperature, the partition of calcium and phosphorus in milk stored at 3 °C would be expected to revert to the original state on warming the milk to 20 °C. This possibility was tested by analysing the following ultrafiltrates from one milk: (1) ultrafiltrate prepared from fresh milk at 20 °C, (2) ultrafiltrate prepared at 3 °C after storing the milk for 24 h at 3 °C, (3) ultrafiltrate prepared at 3 °C after storing the milk for 48 h at 3 °C, and (4) ultrafiltrate prepared at 20 °C after storing the milk for 48 h at 3 °C followed by 12 h at 20 °C. Ultrafiltrates (2) and (3) were identical in composition and both differed from ultrafiltrate (1) in the way expected from the results in Table 6. Ultrafiltrate (4), on the other hand, had the same composition as ultrafiltrate (1) showing that the changes induced at 3° were reversible on warming the milk to 20 °C. Further evidence of this reversibility will be presented in the section dealing with dialysis.

Temperature-associated changes in the composition of the aqueous phase of milk opposite to those just described have been reported by Schwarz & Mumm (1948) and Fricker (1958). According to these authors, when milk is stored at 5 °C for 24–48 h there is a decrease in the concentrations of soluble calcium and phosphorus, a change that is not reversed when the milk is subsequently heated for 30 min at 50 °C. However, the concentrations of calcium, phosphorus and nitrogen in the ultrafiltrates obtained in both of these investigations are different from what one would expect in the aqueous phase of milk. These anomalies appear to have arisen in one instance from blocking and sieving effects as a consequence of using a very high pressure and a membrane with small pores, and in the other instance from the presence of protein in the ultrafiltrate as a result of using a membrane of too great a porosity.

The effect of variations in the method of dialysis on the composition of diffusate

Effect of varying the water to milk ratio on the composition of diffusate

Diffusates were prepared from a sample of bulk milk using water to milk ratios of 1:25, 1:50, 1:100 and 1:200, and with a dialysis period of 48 h at 3 °C. Pairs of Visking tubes each containing 40 ml of water were immersed in the appropriate volume of milk; at the end of the dialysis, each tube contained about 25 ml of diffusate, i.e. about 15 ml of water were lost by osmosis. The results (Table 7) show that the diffusates were very similar in composition. Similar experiments but with dialysis periods of 24 and 72 h confirmed these results. The fact that there was little difference in the composition of the diffusates obtained by using the extreme ratios of 1:25 and 1:200 suggests that even when 25 volumes of milk are diluted with 1 volume of water no change occurs in the partition of the milk constituents between the disperse and aqueous phases.

As a result of these experiments, it was decided to adhere to the water to milk ratio of 1:50 used by Pyne (1940) for preparing diffusate. Proof that the dilution of 50 volumes of milk with 1 volume of water has no effect on the composition of milk other than simple dilution, for which allowance can be made, was obtained by preparing ultrafiltrate at 3 °C from milk and diluted milk, both having been stored for 48 h at 3 °C. (Any changes in the partition of the milk constituents between the disperse and aqueous phases resulting from the dilution would be expected to be complete within 48 h at 3 °C.) The composition of the ultrafiltrate from the diluted milk, when corrected for the dilution, was identical with that of the ultrafiltrate from the undiluted milk. The same result was obtained when ultrafiltrate from milk and diluted milk was prepared at 20 °C immediately after the dilution.

Table 7. *Effect of varying the water to milk ratio on the composition of diffusate from separated milk*

	Water to milk ratio			
	1:25	1:50	1:100	1:200
	mg/100 g milk			
Total calcium	43·6	43·6	43·3	43·4
Ionized calcium	13·9	13·7	13·7	13·3
Magnesium	8·2	8·1	8·1	8·1
Sodium	43	44	44	43
Potassium	141	146	144	144
Total phosphorus	45·8	45·9	45·4	45·7
Inorganic phosphorus	36·4	36·5	35·9	36·2
Citric acid	166	174	168	169
Chloride	102·0	104·9	104·4	104·9
Total nitrogen	24·4	25·2	24·7	25·1
Lactose (anhydrous)	4780	4860	4810	4830
pH	6·84	6·83	6·88	6·90

Effect of varying the duration of dialysis on the composition of diffusate

Diffusates were prepared at 3 °C from a bulk milk and milk from an individual cow using a water to milk ratio of 1:50 and dialysis periods of 24, 48 and 72 h (Table 8). The diffusates obtained after dialysis for 48 and 72 h were identical in composition, but the diffusate obtained after dialysis for 24 h contained slightly less total calcium, citric acid and lactose. It was concluded therefore that in this method of static dialysis at 3 °C, a dialysis period of 48 h was necessary for equilibrium to be established between the diffusate and the aqueous phase of milk.

The results in Table 8 show also that, as in ultrafiltration (Table 3), there was incomplete passage of sodium and potassium through the membrane and that the chloride content of the milk, calculated from the concentration in diffusate, exceeded the value obtained by direct determination. These results, together with the difference between the pH of the diffusates and the milk, may be evidence of a Donnan effect, though the deficiency of sodium and potassium could be caused also by the presence of protein-bound sodium and potassium in milk. The incomplete recovery of citric acid in the diffusates cannot be ascribed to a sieving effect, as any anomalies arising from this phenomenon would be eliminated on the attainment of equilibrium. It

would appear that some of the citric acid in milk, in the present instance about 6% on average, is in the disperse phase. Even although lactose equilibrium was not complete after 24 h dialysis, the lactose content of the 24 h diffusate indicated a concentration in milk greater than the directly determined concentration; the lactose contents of the 48 and 72 h diffusates indicated an even greater discrepancy. In the absence of sieving and Donnan effects, these differences may be an indication of bound water in milk. The close agreement between the concentration of N.P.N. in the milk and the concentration of diffusible nitrogen shows that Visking tubing, like Cellophane (P.T. 300), has a porosity ideal for retaining the milk proteins.

No doubt equilibrium between diffusate and the aqueous phase of milk would be reached in less than 48 h at 3 °C if the milk were stirred, but to keep the dialysis technique as simple as possible a dialysis period of 48 h without stirring was retained as the standard method of preparing diffusate.

Table 8. *Effect of varying the duration of dialysis at* 3 °C *on the composition of diffusate from separated milk (average results from two milks)*

	Duration of dialysis, h			
	24	48	72	Total in milk
	mg/100 g milk			
Total calcium	42·0	42·9	43·0	128·3
Ionized calcium	13·8	13·8	13·8	—
Magnesium	8·4	8·6	8·4	12·8
Sodium	48	46	47	49
Potassium	148	148	148	155
Total phosphorus	52·3	52·5	52·4	106·1
Inorganic phosphorus	37·1	37·4	37·4	—
Citric acid	154	158	158	168
Chloride	105·7	105·2	104·7	101·4
Total nitrogen	28·4	28·4	28·7	28·9*
Lactose (anhydrous)	4790	4870	4890	4740
pH	6·84	6·86	6·84	6·72

* Non-protein nitrogen, not total nitrogen

Reproducibility of the method of preparing diffusate

It is evident from the results in Tables 7 and 8 that the dialysis method can give very reproducible values for the soluble constituents of milk. Nevertheless, the reproducibility of the method was tested by analysing diffusates prepared from a milk with samples of Visking tubing from the same batch and from three different production batches. The diffusates were identical in composition. Any variation in the porosity of Visking tubing within one batch or among different batches was evidently insufficient to influence the composition of the diffusates.

Effect of temperature on the composition of milk diffusate

Ultrafiltration experiments showed that when milk is stored for 24–72 h at 3 °C there is an increase in the concentrations of total calcium, ionized calcium and phosphorus in the aqueous phase of the milk (Table 6), and that the concentrations of these constituents revert to the original values on subsequently warming the milk

to 20 °C. Confirmation of these changes was sought by comparing the composition of diffusates prepared at 3 and 20 °C. To prepare diffusate at 20 °C it is necessary to add a preservative to the milk; chloroform (0·25 ml per 100 ml milk) was found to be very satisfactory. Milk containing this concentration of chloroform, provided it is mixed occasionally to disperse and dissolve the chloroform, can be kept for at least 48 h at 20 °C with no detectable change in composition other than a slight increase in the level of proteose-peptone nitrogen and a very small degree of hydrolysis of ester phosphorus compounds.

Table 9. *Effect of temperature on the composition of diffusate obtained by dialysis of separated milk under different conditions (average results from two milks)*

	Dialysis conditions				
	24 h at 20 °C	24 h at 3 °C	48 h at 20 °C	48 h at 3 °C	24 h at 20 °C*
	mg/100 g milk				
Total calcium	37·9	40·1	37·8	41·2	38·0
Ionized calcium	12·2	13·2	12·0	12·9	12·0
Magnesium	7·8	7·7	7·7	7·9	7·7
Sodium	58	58	58	60	59
Potassium	133	132	133	133	132
Total phosphorus	38·2	39·7	37·8	39·2	37·9
Inorganic phosphorus	31·8	32·8	32·0	32·6	32·1
Citric acid	173	170	177	175	175
Chloride	122·4	123·0	122·2	121·9	122·6
Total nitrogen	25·0	24·5	25·6	25·2	25·0
Lactose (anhydrous)	4800	4740	4800	4780	4810
pH	6·94	6·96	6·94	6·97	6·90

* Milk previously stored 48 h at 3 °C

Table 10. *Reversion at 20 °C of changes induced in the composition of diffusate by storing separated milk at 3 °C*

	Dialysis conditions		
	24 h at 20 °C	24h at 3 °C	24 h at 3 °C plus 24 h at 20° C
	mg/100 g milk		
Total calcium	35·7	39·8	35·7
Ionized calcium	11·9	13·6	11·6
Total phosphorus	47·5	49·6	48·4
Inorganic phosphorus	35·9	36·9	36·0
pH	6·78	6·90	6·80

Five diffusates were prepared from a herd bulk milk and also from milk from an individual cow under the following conditions of dialysis: 24 h at 20 °C, 24 h at 3 °C, 48 h at 20 °C, 48 h at 3 °C, and 24 h at 20 °C using milk that had been stored for 48 h at 3 °C. In this instance, chloroform was used in all the dialyses. The results in Table 9 show that there was an increase in the concentrations of total soluble calcium, ionized calcium and soluble phosphorus, and in the pH of the aqueous phase,

when milk was kept at 3 °C. A comparison of the values in the first and last columns again shows that these changes are reversible. Further evidence of this reversibility was obtained by comparing the composition of diffusates prepared under the following conditions from milk preserved with chloroform: (1) 24 h at 20°C, (2) 24 h at 3 °C, and (3) 24 h at 3° plus 24 h at 20 °C, i.e. diffusate (3) was obtained by keeping the cylinder of milk with the immersed Visking tube at 3 °C for 24 h and then at 20 °C for a further 24 h (Table 10).

Comparison of the ultrafiltration and dialysis methods for determining the concentrations of soluble constituents in milk

From the separate examination of the ultrafiltration and dialysis methods it would appear that the differences in the composition of ultrafiltrate prepared at 20 °C and diffusate prepared at 3 °C (Table 1) are due partly to the temperature difference and partly to the sieving phenomenon in ultrafiltration. To verify these deductions it was necessary to compare the composition of ultrafiltrates and diffusates prepared from the same milk. For this comparison, ultrafiltrates were prepared at 20 °C using Cellophane sheet and Visking tubing, and diffusates were prepared at 20 and 3 °C. A pressure of 38 cm of mercury was used for ultrafiltration, and a water to milk ratio of 1:50 and an equilibration period of 48 h were used in the dialyses. In dialysis at 20 °C chloroform was added to the milk.

Table 11. *Composition of sera obtained by ultrafiltration at* 20° C *and dialysis at* 20 *and* 3 °C *of separated milk* (*average results from three milks*)

	Ultrafiltrate		Diffusate		
	Cellophane 20 °C	Visking 20 °C	Visking 20 °C	Visking 3 °C	Total in milk
	mg/100 g milk				
Total calcium	38·7	39·4	39·8	43·2	115·9
Ionized calcium	11·7	11·8	11·8	13·5	—
Magnesium	8·3	8·3	8·0	8·2	11·4
Sodium	46	46	47	47	48
Potassium	139	140	142	142	149
Total phosphorus	39·4	39·6	40·2	41·4	87·5
Inorganic phosphorus	32·7	33·1	33·9	34·6	—
Citric acid	162	166	168	166	175
Chloride	104·3	104·6	104·9	104·8	102·6
Total nitrogen	22·5	21·3	22·2	21·6	21·9*
Lactose (anhydrous)	4790	4837	4923	4947	4810
pH	6·82	6·80	6·82	6·89	6·77

* Non-protein nitrogen, not total nitrogen

The results in the first two columns of Table 11 show the effect of the difference in porosity of the Cellophane and Visking membranes, and hence in sieving effect, on the composition of ultrafiltrate. Differences between corresponding values are very small and, with the exception of those for total calcium, citric acid and lactose, are of little significance. A comparison of the values in the second and third columns shows that, at 20° C with the same membrane, the ultrafiltration and dialysis procedures gave sera almost identical in composition. It is evident from these results

that the operation and variation of the sieving phenomenon were of little significance apart from having a small effect on the concentration of lactose, citric acid and total calcium. The same conclusion could be drawn from the composition of corresponding ultrafiltrates and diffusate prepared at 3 °C. However, when ultrafiltrate or diffusate is prepared at different temperatures, the composition of the serum varies appreciably. This can be seen by comparing the values in the fourth column of Table 11 with those in the first three and also from the results in Tables 6, 9 and 10.

It is clear that dialysis at 3 °C is not a satisfactory procedure for determining the partition at room temperature of the constituents of milk between the disperse and aqueous phases. It is also clear that ultrafiltration through Cellophane sheet or Visking tubing at 20 °C, at a pressure sufficient to give an adequate rate of filtration, is not an entirely satisfactory method of preparing a serum for the determination of some soluble constituents because of the sieving effect. Attempts made to minimize or obviate the sieving effect by increasing the porosity of Cellophane by treating it with a zinc chloride solution (McBain & Stuewer, 1936; Seymour, 1940) were unsuccessful because of sloughing of the membrane surface. It was decided therefore that of the procedures examined for isolating the aqueous phase of milk, dialysis at 20 °C of milk containing chloroform was open to least criticism.

The validity of values for the soluble constituents of milk calculated from the composition of diffusate obtained at 20 °C

It now remained to determine whether the composition of diffusate obtained at 20 °C gives a true picture of the composition of the aqueous phase of milk. The concentrations of ions in a diffusate from a protein-containing fluid like milk might be expected to differ slightly from the concentrations in the aqueous phase because of a Donnan membrane equilibrium. The implication of such an equilibrium is that the net negative charge of the caseinate micelles restrained by the membrane will be compensated, partially by an excess of cations and partially by a deficiency of anions, and that the diffusate will be deficient in cations and enriched with anions. The results in Table 11 suggest that milk diffusate is deficient in sodium, potassium and hydrogen ions and enriched with chloride ions. As already pointed out, however, the deficiency of sodium and potassium may indicate the presence of protein-bound sodium and potassium in milk; it is unlikely that a significant part of this deficiency can be attributed to adsorption or retention by the Visking tubing. The results in Table 11 show also that the lactose content of milk, when calculated from the concentration in diffusate, exceeds the concentration found by direct analysis. The most likely explanation of this result is that some of the water in milk is not acting as a solvent but is associated with the protein as bound water. If this is so, allowance must be made for bound water when calculating the concentrations of soluble constituents in milk from the composition of diffusate.

An attempt was made to determine to what degree the composition of diffusate differs from that of the aqueous phase of milk, because of a Donnan membrane equilibrium, by comparing the composition of diffusate obtained from milk at 20 °C and the composition of centrifuge serum and rennet whey, two sera prepared without the use of a membrane. The effect of making allowance for bound water when calcu-

lating the concentrations of soluble constituents in milk from the composition of these sera was also examined. The composition of the three sera (Table 12) suggests that all are affected to some degree by a Donnan effect. With whey and centrifuge serum this effect would be regarded as an 'internal' one in accordance with the modern view of the Donnan equilibrium (Overbeek, 1956). However, when a correction is made for bound water on the basis of the lactose values, the concentration of chloride in milk calculated from the sera values is the same as the directly determined value. Although this does not necessarily indicate the absence of a Donnan equilibrium (Overbeek, 1956), it seems likely that any influence of a Donnan effect on the composition of the sera is very small. The sera values for sodium and potassium when corrected for bound water are probably therefore a true measure of the concentrations of these ions in solution in milk. The difference between these values and the corresponding directly determined values are in all probability a measure of the amounts of sodium and potassium bound to protein.

Table 12. *Composition of diffusate* (20 °C), *whey and centrifuge serum from separated milk* (*average results, uncorrected and corrected for bound water, from two milks*)

	Diffusate (20 °C)		Whey		Centrifuge serum		Total in milk
	Uncorr.	Corr.	Uncorr.	Corr.	Uncorr.	Corr.	
	mg/100 g milk						
Total calcium	39·0	38·1	40·7	39·9	41·4	40·9	114·2
Ionized calcium	12·0	11·7	11·8	11·6	12·0	11·9	—
Magnesium	7·6	7·4	8·0	7·8	8·2	8·1	11·0
Sodium	47	46	48	47	48	47	50
Potassium	140	137	146	143	143	141	148
Total phosphorus	38·6	37·7	38·2	37·4	38·4	37·9	84·8
Inorganic phosphorus	32·6	31·8	31·4	30·8	32·2	31·8	—
Citric acid	160	156	155	152	156	154	166
Chloride	109·2	106·5	108·4	106·2	106·9	105·6	106·3
Total nitrogen	21·2	20·7	127·2	124·6	112·1	110·7	21·4*
Casein nitrogen	0	0	22·0†	21·6	6·9	6·8	364
Lactose (anhydrous)	4920	4800	4900	4800	4860	4800	4800
pH	6·82	—	6·78	—	6·81	—	6·78

* Non-protein nitrogen, not total nitrogen
† Mostly 'whey proteose' derived from casein

When allowance is made for the fact that whey and centrifuge serum contain all the soluble nitrogenous components of milk and a trace of the calcium caseinate complex, and also 'whey proteose' in the case of whey, the corrected diffusate, whey and centrifuge serum values are similar. That this is so, despite the completely different preparative methods, is strong presumptive evidence that the composition of diffusate prepared at 20 °C is the same as that of the aqueous phase of milk at 20 °C.

The corrected diffusate values in Table 12 show that some calcium, magnesium, sodium, potassium, phosphorus and citric acid are present in the disperse phase of milk. These 'colloidal' fractions should dissolve when milk is sufficiently acidified. To demonstrate this, diffusate was prepared at 20 °C from control milk (pH 6·77) and from portions of the same milk acidified with hydrochloric acid to pH values of 5·60 and 4·57, respectively. The results, corrected for bound water (Table 13), show

that as the pH of the milk was lowered there were progressive increases in the concentrations of calcium, magnesium, sodium, potassium, phosphorus and citric acid in solution; at a pH of 4·57, approximately the isoelectric point of casein, all the sodium and potassium and almost all the calcium, magnesium and citric acid were in the aqueous phase. The increase in the concentration of total soluble phosphorus, and of inorganic phosphorus, is a measure of the colloidal phosphate that dissolved; the 27% of the total phosphorus that remained in the disperse phase at pH 4·57 would be mainly the phosphorus of the casein molecule.

Table 13. *Composition of diffusate* (20 °C) *from separated milk* (pH 6·77) *and from portions of the milk acidified with hydrochloric acid* (*average results, corrected for bound water, from two milks with the same initial* pH)

	pH of milk						
	6·77		5·60		4·57		Total in milk
	mg/100 g milk	% of total	mg/100 g milk	% of total	mg/100 g milk	% of total	mg/100 g milk
Total calcium	36·0	30·5	80·5	68·1	115·0	97·3	118·2
Magnesium	7·6	65·5	9·1	78·5	11·1	95·7	11·6
Sodium	49	98·0	52	104·0	51	102·0	50
Potassium	140	93·9	146	98·0	151	101·4	149
Total phosphorus	43·4	45·6	64·0	67·2	69·5	73·0	95·2
Inorganic phosphorus	34·7	—	53·0	—	60·5	—	—
Citric acid	158	93·5	165	97·6	166	98·2	169
Total nitrogen	28·6	—	28·6	—	28·7	—	30·1*
Lactose (anhydrous)	4790	—	4790	—	4790	—	4790
pH	6·81	—	5·78	—	4·62	—	6·77

* Non-protein nitrogen, not total nitrogen

CONCLUSIONS

Although the porosity of Cellophane sheet and Visking tubing is ideal for the ultrafiltration of milk, in that practically all the protein is retained by these membranes, their permeability to lactose and citric acid varies slightly with the applied pressure. Since part of the soluble citric acid in milk is in the form of calcium citrate, the concentration of calcium in ultrafiltrate, as well as that of lactose and of citric acid, will be influenced by the sieving effect and hence will differ from the concentrations in the aqueous phase of milk. The empirical nature of the lactose content of ultrafiltrate makes it difficult to apply the necessary correction for bound water when calculating the concentrations of soluble constituents in milk from the composition of ultrafiltrate. Nevertheless, the ultrafiltration of milk at 20 °C through Cellophane (P.T. 300) at a pressure of 38 cm of mercury will give a serum whose composition is reasonably close to that of the aqueous phase of milk, and should be satisfactory for measuring changes in the composition of the aqueous phase resulting from a particular treatment of milk. Ultrafiltration at 20 °C with Visking tubing by the technique described, although giving a serum whose composition is even closer to that of the aqueous phase of milk, is not convenient for routine use.

There do not appear to be any reasons for supposing that diffusate, obtained by the dialysis at 20 °C for 48 h of 50 volumes of milk containing 0·25% of chloroform

against 1 volume of water in a Visking tube, does not truly represent the aqueous phase of milk. An accurate allowance for bound water, based on the relation between the lactose content of milk directly determined and that calculated from the level in diffusate, can therefore be made when expressing the concentrations of constituents in diffusate as concentrations in milk. When allowance is made for bound water, the concentration of chloride in diffusate does not appear to be governed to a significant extent by a Donnan equilibrium. Also, the fact that the difference between the pH of diffusate and the pH of milk is small may be further evidence that the Donnan effect is also small. These findings do not exclude the possibility that the net negative charge of the restrained caseinate micelles is compensated almost entirely by an excess of cations; according to Overbeek (1956), this type of Donnan equilibrium, whether 'membrane' or 'internal', would exist if milk can be regarded as a suspension of highly charged particles in a serum of low ionic strength. However, it seems likely that any Donnan effect in milk is very small, and that the deficiency of sodium and potassium in diffusate can be accepted as evidence that, on average, about 5% of the sodium and about 6% of the potassium in milk are combined with protein.

The preparation of a diffusate from a biological fluid is sometimes conducted at a low temperature to prevent any bacterial decomposition during the equilibration period, for example see Pyne (1940). It has been found in the present investigation that when diffusate is prepared from milk at 3°C, the diffusate contains more total calcium, ionized calcium and phosphorus than a diffusate prepared at 20°C. The reason for these differences appears to be that at 3°C some of the calcium and phosphorus associated with the casein pass into the aqueous phase. This view is supported by the fact that these temperature-associated changes are reversible. Thus if it is desired to determine the composition of the aqueous phase of milk, or the partition of milk constituents between the disperse and aqueous phases, at normal ambient temperature it is essential that the diffusate be prepared at 20°C. As the period required for equilibration was found to be 48 h it is necessary to add a preservative to the milk when dialysis is conducted at 20°C. Chloroform, 0·25 ml per 100 ml of milk, is a suitable preservative.

In an earlier investigation by the present authors (White & Davies, 1958), no correction for bound water was applied in calculating the partition of milk constituents between the disperse and aqueous phases from the composition of ultrafiltrate obtained at 20°C. Since only about 2% of the water in milk appears to be bound, i.e. approximately 0·5 g/l g of protein (cf. de Kadt & van Minnen, 1943; von Hippel & Waugh, 1955), the omission of this correction introduces only a small error and partially compensates for the effects of the sieving phenomenon. Thus the previous results obtained by ultrafiltration are in good agreement with those obtained by dialysis at 20°C, except that only about 6% of the citric acid in milk, instead of 10–13%, is now believed to be in the disperse phase.

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REFERENCES

CLARK, L. C. (1951). *J. Lab. clin. Med.* **37**, 481.
DE KADT, G. S. & VAN MINNEN, G. (1943). *Rec. Trav. chim. Pays-Bas*, **62**, 257.
FERRY, J. D. (1936). *Chem. Rev.* **18**, 373.
FRICKER, A. (1958). *Dtsch. Molkereiztg*, **79**, 1553.
LAMPITT, L. H. & BUSHILL, J. H. (1933). *Biochem. J.* **27**, 711.
MCBAIN, J. W. & STUEWER, R. F. (1936). *J. phys. Chem.* **40**, 1157.
OVERBEEK, J. T. G. (1956). *Progress in Biophysics and Biophysical Chemistry*, vol. 6, p. 57, ed. Butler, J. A. V. London: Pergamon Press.
PYNE, G. T. (1940). *J. Dairy Res.* **11**, 292.
SCHWARZ, G. & MUMM, H. (1948). *Molkereiztg, Hildesh.* **2**, 79.
SEYMOUR, W. B. (1940). *J. biol. Chem.* **134**, 701.
VAN DER HAVE, A. J. & MULDER, H. (1957). *Ned. melk- en Zuiveltijdschr.* **11**, 128.
VON HIPPEL, P. H. & WAUGH, D. F. (1955). *J. Amer. chem. Soc.* **77**, 4311.
WHITE, J. C. D. & DAVIES, D. T. (1958). *J. Dairy Res.* **25**, 236.

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Bulk feeds for milk production

I. The influence of level of concentrate feeding in addition to silage and hay on milk yield and milk composition

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SUMMARY. Two experiments were carried out, Expt. 1 with twelve cows in a 4 × 4 Latin square and Expt. 2 with twenty-six cows in two groups which spent 11 weeks on the experimental rations. In both experiments hay and silage were fed *ad lib.* In Expt. 1 four levels of concentrate feeding were offered, $1\frac{1}{4}$, $2\frac{1}{2}$, $3\frac{3}{4}$ and 5 lb/gal, and in Expt. 2 two levels, $1\frac{1}{4}$ and 5 lb/gal. The levels of feeding as a percentage of Woodman's standards for starch equivalent (S.E.) ranged from 82 to 125 % in Expt. 1 and from 83 to 116 % in Expt. 2; for digestible crude protein (D.C.P.) the corresponding figures were 70–100 % and 105–107 %. When concentrate consumption increased by 1 lb dry matter the consumption of bulk feeds was reduced by 0·18–0·22 lb dry matter but the total fibre intake remained constant. Milk yield showed small but significant responses to level of feeding. Average responses per 1 lb S.E. were 0·77 lb milk in Expt. 1 and 0·61 lb milk in Expt. 2. Total lactation yields of the cows in Expt. 2 were unaffected by the experimental treatments. The production S.E. fed per gallon of milk ranged from 1·71 to 3·72 lb in Expt. 1 and from 1·92 to 3·27 lb in Expt 2. Production D.C.P. ranged from 0·37 to 0·63 lb and from 0·67 to 0·70 lb, respectively.

High levels of feeding increased the S.N.F. and protein contents of the milk but did not affect other constituents. The responses in S.N.F. were lower than in some other experiments and some cows showed no response. The results are discussed with reference to the experimental methods and also with reference to the yield responses compared with published data, and to the practical importance of the high production obtained with low usage of concentrate feeds.

Economic trends in Great Britain have emphasized the importance of a full appreciation of the extent to which grass can contribute to the winter feeding of dairy cows, and of the influence of various combinations of grass and concentrate feeds on the economy of production and on the composition of milk. For this reason work further to that described earlier by Holmes, Waite, MacLusky & Watson (1956) Holmes, Reid, MacLusky, Waite & Watson (1957) and Reid & Holmes (1956) has been done at Wye College.

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EXPERIMENTAL

Two experiments were carried out, Expt. 1 in 1955–6 and Expt. 2 in 1956–7. In the first a balanced Latin square design, with twelve cows, was adopted, and in the second a group comparison with twenty-six cows was made.

Management

The cows were housed in two covered yards equipped with individual ties and separated feeding troughs. Each cow was randomly allocated to a tie and concentrates and roughages were fed at this tie on three occasions totalling about 6 h each day. Water was freely available when the cows were not tied. The yards were littered daily with straw, a little of which may have been consumed by the cows. The cows received a modest steaming-up allowance of up to 100 lb concentrates per cow and were fed in the early part of the lactation according to normal standards with kale, hay and concentrates. They were housed and introduced to the experimental routine and to the concentrate mixture, fed at a standard rate of 3½ lb/gal, for 4 weeks before the experiments began. In the introductory period for Expt. 1 hay and silage were fed *ad lib.* at the same times as they were fed in the experimental period. In Expt. 2 kale and hay were fed *ad lib.* during the introductory period and silage was introduced during the week when cows were changed to the experimental levels of concentrate feeding. At the end of Expt. 1 the cows were gradually changed on to grazing with no supplementary concentrates. There was a control period of 2 weeks at the end of Expt. 2 when the cows were fed on hay and mangolds and were given concentrates at 3½ lb/gal before they were gradually changed on to pasture. Again no concentrates were fed when the cows were fully at pasture.

Records and sampling

The feeds and feed refusals were individually weighed for each cow on four consecutive days per week during the pre-experimental, experimental and post-experimental periods. On the remaining 3 days per week concentrates were weighed and rationed individually, but hay and silage were not weighed individually, although the experimental group of cows received the same total weight of each feed as in the 4 days when individual feeding was practised. Samples of hay, silage and concentrates were taken daily. The moisture content of the silage was determined on four samples daily. The moisture content of the other feedingstuffs and the crude fibre, crude protein, ether extract and ash contents of composite samples of each constituent of the ration were determined weekly by standard methods (Fertilizer and Feeding-Stuff Regulations, 1955), the Markham apparatus being used for Kjeldahl distillations. Calcium was determined by flame photometry as described by Powell (1953) and phosphorus by the method of Rockstein & Herron (1951).

The cows were weighed daily at 10 a.m. on 5 consecutive days each week. Milking took place twice daily and yields were recorded to the nearest ½ lb at each milking. A daily composite milk sample, representative of the 24 h production of each cow, was taken at the end of each week. This was analysed for fat by the Gerber method (British Standards Institution, 1955), total solids by a standard gravimetric pro-

cedure (British Standards Institution, 1951) using 1 g of milk, and lactose iodometrically by a modification of the method of Hinton & Macara (1927). Total nitrogen and casein nitrogen were determined by the method of Rowland (1938), chloride by the wet oxidation method (Davies, 1932), and calcium and phosphorus volumetrically after extraction from the ash.

Level of feeding

The quantities of S.E. and D.C.P. fed to each cow were calculated for all rations used in both experiments, the S.E. and D.C.P. values for concentrates and kale being taken from Woodman (1954). Those for silage and hay were calculated from the regression equations of Dijkstra (1954) and Watson (1939), respectively. The level of feeding on the basis of Woodman's standards (using the higher production requirement of 0·6 lb D.C.P./gal) was calculated for each cow for each period from the estimated consumption and from the average live weight and milk production per cow, as described by Holmes *et al.* (1956).

EXPERIMENT 1

This experiment was of a Latin square design (Cochran, Autrey & Cannon, 1941) with three groups of four Ayrshire cows, and ran without interruption from 5 December 1955 to 26 March 1956. After 4 preliminary weeks each cow spent four periods, each of 4 weeks, on each of the treatments in a sequence indicated by the design. On 5 December, when the experimental periods began, the cows had been in milk for 44–93 (mean 71) days. Hay and silage were fed *ad lib.* and concentrates at $1\frac{1}{4}$ (treatment A), $2\frac{1}{2}$ (treatment B), $3\frac{3}{4}$ (treatment C), and 5 lb (treatment D) per gallon.

The average composition of all the feedingstuffs is shown in Table 1, together with

Table 1. *The chemical composition and nutritive value of the feedingstuffs used in Expts* 1 *and* 2

	Dry matter content as fed, %	% of the dry matter							
		Crude protein	Crude fibre	Ether extract	Ash	Ca	P	D.C.P.	S.E.
Silage									
Expt. 1	22·4	11·7	34·7	2·95	—	0·66	0·30	7·1	44
Expt. 2	22·0	12·4	33·9	4·93	8·8	0·84	0·23	7·7	48
Hay									
Expt. 1(*a*)	84·7	8·9	41·4	1·48	—	0·46	0·20	4·1	28·5
Expt. 1(*b*)	83·9	11·1	33·3	1·40	—	0·51	0·38	5·8	36·0
Expt. 2	85·1	16·3	30·9	3·10	10·7	1·26	0·31	9·9	35·8
Concentrate									
Expt. 1	87·0	14·3	7·5	1·57	—	0·57	0·42	12·3	78·5
Expt. 2									
High	84·3	14·2	5·8	3·11	8·6	0·68	0·58	12·3	78·5
Low	85·1	32·2	8·2	3·11	8·5	0·51	0·45	29·0	79·0
Kale									
Expt. 2	16·8	10·3	19·0	5·2	10·5	1·71	0·31	10·0	65·0

The mean pH values of the silage were 5·2 in Expt. 1 and 4·3 in Expt. 2.

the calculated D.C.P. and S.E. values. The concentrate mixture included the following constituents (% by weight): barley meal 43, dried sugar-beet pulp 32, flaked maize 10, decorticated ground-nut cake 5, soya-bean meal 5, molassine meal 3, steamed bone flour 1, calcined magnesite 1. It was low in protein content but it was expected that adequate protein for the levels of milk production expected would be derived from it and from the silage and hay. Allowances of concentrates were adjusted at the beginning of each period, according to the equalized feeding principle described by Lucas (1943), the feed for each of the twelve cows being adjusted according to the average decline in milk yield of the group and the particular level of feeding of each cow for that period. The third period coincided with a very cold spell of weather to which reference is made later. After the 16 experimental weeks the cows were gradually introduced to spring grazing but because of the late spring they were not fully at grass, and were receiving supplementary feed, until mid-May.

RESULTS

The results reported relate to the last 3 weeks of each period. The significance of the treatment differences was tested by analysis of variance.

Feed consumption

The mean daily intakes of silage, hay and concentrates as dry matter, and the estimated consumption of D.C.P. and S.E., are given in Table 2 which also shows the estimated level of feeding as a percentage of Woodman's standards.

The treatments had a significant effect on the total feed consumption, which increased linearly with increased concentrate feeding. There was also a significant decline in total dry-matter consumption per day from 32·4 lb in period 1 to 28 lb in period 4, largely due to the reduction in concentrates offered as milk yield declined. Large and significant differences in the daily consumption of D.C.P. and S.E. were allied with these differences in total food consumption. While the average D.C.P. and S.E. in the feed increased from 7·2 and 46 %, respectively, on treatment A to 8·7 and 58 % on treatment D, and the fibre content decreased from 31 to 23 %, the total fibre intake remained virtually constant at 7·7–7·9 lb/day.

The level of feeding of D.C.P. ranged from 70 to 100 % of the Woodman standard while the S.E. level increased from 82 to 125 % for treatments A to D. The level of feeding remained almost constant from period to period, confirming the efficacy of the method of feed allocation adopted.

Table 2 shows also that the intake of grass dry matter from hay and silage ranged only from 21·2 lb/day on treatment A, to 19 lb on treatment D. The treatments had no significant effect on silage intake, but there was a small but significant decrease in hay intake with increased concentrate feeding. On all treatments and in each period the consumption of silage dry matter increased the higher the dry-matter content of the silage (within the range 17–30 %), and an increase of 1 lb in the intake of silage dry matter increased the total dry-matter intake by approximately 0·9 lb.

Average live weights are included in Table 2. From these and the feed consumptions it can be calculated that the appetite, expressed as the dry-matter intake as

a percentage of live weight, increased from 2·26 on treatment A to 3·03 on treatment D. There was a general decline in appetite from period to period associated mainly with the reduction in concentrate allowance.

Table 2. *Feed consumption, animal production and efficiency of feed conversion in Expt.* 1

Treatment	Pre-experimental 3 weeks	Experimental treatments A	B	C	D	Standard error of the treatment mean	At grazing for 3 weeks (after an interval of 7 changeover weeks)
Feed consumption, lb dry matter per cow per day							
Silage	14·9	12·9	12·5	12·3	12·3	±0·22	—
Hay	6·5	8·3	7·5	7·2	6·7	±0·21	—
Concentrates	12·6	4·0	7·9	11·9	15·8	—	—
Total	34·0	25·2	27·9	31·4	34·8	±0·43	—
D.C.P.	2·82	1·81	2·21	2·69	3·02	±0·14	—
S.E.	18·5	11·5	14·2	17·2	20·2	±0·25	—
% Woodman's standards							
D.C.P.	81	70	82	93	100	—	—
S.E.	110	82	97	113	125	—	—
Animal production, lb per cow							
Milk/day	43·7	30·0	31·7	34·5	36·5	±0·56	33·5
Live weight	1148	1111	1120	1132	1147	±3·5	1143
Live-weight gain/day	—	−1·76	−0·64	+0·11	+0·33	±3·5	±1·7
Feed efficiency. Total nutrients including maintenance, lb							
D.C.P./gal	—	0·60	0·70	0·78	0·83	—	—
S.E./gal	—	3·83	4·48	4·99	5·53	—	—
S.E./gal (adjusted*)	—	5·78	5·18	4·66	4·82	—	—
Production nutrients excluding maintenance, lb							
D.C.P./gal	—	0·37	0·48	0·57	0·63	—	—
S.E./gal	—	1·71	2·45	3·12	3·72	—	—
S.E./gal (adjusted*)	—	2·74	3·05	2·93	3·29	—	—

* Adjusted for live-weight changes according to Yates, Boyd & Pettitt (1942) (1 lb live-weight gain = 10 lb milk, 1 lb live-weight loss = 7 lb milk).

Milk yield

The data given in Table 2 show that there was a significant increase in milk yield with increased concentrate feeding. The increase was linear and on average each increase of 1 lb of S.E. fed resulted in an increase of 0·77 lb milk. There was a significant decline in average milk yield from 38·4 lb per cow per day in period 1 to 29·4 lb per cow per day in period 4, associated with advancing lactation. Spring grass produced a marked increase in average yield over period 4 to 33·5 lb in the 3-week period when the cows were fully at grass. The increase in milk yield on grazing depended on the previous treatment, being 5·4 lb/day on treatments A and B compared with 3·8 lb on treatment C and 0·6 lb on treatment D.

Live weight

Live weight increased significantly with increasing feed supply. Differences between periods were not significant. The treatment effect was confirmed by the mean regression of daily live weight on time for each cow within each treatment, which showed a range from a loss of 1·76 lb per cow per day on treatment A to a gain of 0·33 lb on treatment D. Visual assessments of body condition at the end of each period showed small but consistent changes in agreement with the changes in live weight.

Spring grass caused an initial fall in live weight, after which cows showed appreciable gains in weight from period 4 up to 4 June of 1·7, 2·1, 1·6, 1·5 lb per cow per day for cows which were on treatments A, B, C and D, respectively, in period 4.

Efficiency of food conversion

Values for the total S.E. and production S.E. above maintenance, fed per gallon of milk, are included in Table 2. By both measures there was an increase in the requirement per gallon with increased level of feeding. The apparent requirement for production S.E. varied from far below the normal standard of 2·6 lb/gal to well above it. This was associated, however, with changes in milk yield, live weight and milk composition. Adjusted figures, when allowance was made for live-weight changes as suggested by Yates, Boyd & Pettitt (1942) are included in Table 2. They show that the total S.E. required per gallon was minimal with treatment C in which $3\frac{3}{4}$ lb concentrates were fed per gallon, and that the difference in production S.E. per gallon between treatments was also narrowed by the adjustment.

Similar calculations for protein showed that the total D.C.P. fed per gallon increased from 0·6 lb on treatment A to 0·83 lb on treatment D. The quantities of production D.C.P. fed per gallon rose from 0·37 lb/gal on treatment A, a figure well below the recommended standard, to 0·63 lb on treatment D.

Variations in nutrient requirement from period to period were not significant, but the average production S.E. required per gallon was appreciably higher at 3·6 lb in period 3 than in the other periods (2·7–2·9). This period coincided with the coldest spell of the winter with a mean maximum temperature of 35 °F and a mean minimum temperature of 21 °F, some 10 °F lower than in periods 2 and 4. The less efficient food utilization in cold conditions agrees with the findings of Ragsdale, Thompson, Worstell & Brody (1950).

Milk composition

The results for milk composition given in Table 3 show that increased levels of feeding significantly increased the percentages of solids-not-fat (S.N.F.), total protein, casein, ash, calcium and phosphorus. On the other hand the treatments had no significant effect on lactose, chloride or butterfat percentages. The increase in S.N.F. content of milk with increased concentrate feeding was due almost entirely to an increase in protein content. There was also a small but consistent increase in ash and phosphorus contents. There was a decline in average S.N.F. content from 8·69% in period 1 to 8·45% in period 4, which was associated with declines in both the protein and the lactose percentages. Spring grass produced milk of considerably

higher S.N.F. content than that of any of the four levels of concentrates. Cows on treatments A to D in period 4 showed increases in S.N.F. content of their milk of 0·55, 0·48, 0·48 and 0·31, respectively, for the 3-week grazing period. These increases were almost entirely accounted for by increases in the protein content of the milk.

Table 3. *Milk composition* (%) *in Expt.* 1

	Pre-experimental 3 weeks	Experimental treatments A	B	C	D	Standard error of the treatment mean	At grazing for 3 weeks (after 7 weeks)
Fat	4·0	4·01	4·14	3·95	4·10	±0·05	3·89
S.N.F.	8·56	8·42	8·55	8·63	8·71	±0·03	8·91
Total protein*	3·02	2·96	3·07	3·11	3·21	±0·024	3·49
Casein†	2·37	2·29	2·35	2·41	2·50	±0·022	2·70
Lactose (anhydrous)	4·64	4·52	4·52	4·50	4·52	±0·03	4·52
Ash	0·705	0·688	0·703	0·711	0·715	±0·003	0·709
Calcium	0·115	0·113	0·115	0·116	0·117	±0·001	0·117
Phosphorus	0·087	0·082	0·086	0·086	0·089	±0·001	0·092
Chloride	0·099	0·104	0·100	0·101	0·099	—	0·106

* Total N × 6·38. † Casein N × 6·38.

EXPERIMENT 2

This experiment ran from 26 November 1956 to 6 May 1957. In the 4-week preliminary period the same concentrate mixture used in Expt. 1 was fed at 3½ lb/gal with kale and hay *ad lib.*

The twenty-six cows in the experiment were paired according to the milk yield in the preliminary period, and days from calving. One cow of each pair was allotted at random to a 'High' concentrate treatment and the other to a 'Low' concentrate treatment. After 1 week, during which silage replaced kale and the feeding of concentrates at either 5 lb/gal (High) or 1¼ lb/gal (Low) was introduced, each cow remained on the treatment to which it had been allotted for 11 weeks. On 1 January when the experimental period began the cows on the High group had been, on average, 84 days in milk and the cows in the Low group 82 days.

For the High treatment, cows received the concentrate mixture supplied in Expt.1. Cows on the Low treatment received a mixture of the same constituents in different proportions as follows (% by weight): barley meal 17, dried sugar beet pulp 13, flaked maize 4, decorticated ground-nut cake 35, soya-bean meal 26, molassine meal 3, steamed bone flour 1, calcined magnesite 1.

The chemical composition of the feedingstuffs and concentrate mixtures is shown in Table 1. The concentrate allowance of each individual cow at the beginning of the 11-week period was based on her milk yield and the butterfat content in the last 2 weeks of the preliminary period. Subsequent adjustments in concentrate allowance were made at weekly intervals when the ration of each individual cow was adjusted in accordance with the average change in milk yield of her group.

There was some deterioration in the quality of the silage in the last week of the 11-week period and cows were therefore changed fairly rapidly to a diet of 3½ lb of concentrate per gallon (High mixture) with mangolds and hay *ad lib.* After 2 weeks

the cows were gradually changed to pasture so that concentrate and mangold allowances were discontinued at the end of 2 weeks at grass. Hay was still offered and 1 to 2 lb per cow per day was eaten until the sixth week at grazing.

RESULTS

The effect of the treatments in the experimental period was measured both by the results obtained in that period and, for items for which it was not possible to balance the groups, for example, milk composition, by reference to the results given in the pre-experimental period and in the period at grass when both groups were under uniform nutritional conditions. For these items the treatment effect was measured as the difference between the mean result for the pre-experimental and grazing periods and the result for the experimental period. The mean results for each period are shown in Tables 4 and 5.

Feed consumption

It will be seen from Table 4 that the average total dry-matter intake in the introductory period was 31·8 lb/day for the Low group and 33·8 lb for the High group. The introduction of silage and the experimental concentrate levels resulted in average intake figures for the experimental period of 27·3 and 36·1 lb dry matter per cow, both figures being about 2 lb higher than for the corresponding treatments in Expt. 1. This was due to a higher intake of silage and hay dry matter, presumably because of their higher quality, at both levels of feeding. There was only a small decline in feed intake per day during the experimental period, amounting to 1 lb dry matter per cow in both treatments, mainly due to the reduction in concentrate allowances.

The range of feeding of S.E. was similar to but not so wide as that obtained in Expt. 1, 83–116 % of Woodman's standard, but the use of a high-protein concentrate at the Low level, and the higher protein content of the hay used in this experiment, resulted in the cows' on both treatments receiving adequate protein allowances amounting to 105 and 107 % of Woodman's standard. The concentration of S.E. in the dry matter of the two rations was 49 and 57 % and that of D.C.P. was 11·6 and 10·0 % for the Low and High levels, respectively. The fibre content in the dry matter of the rations was 28·8 and 21·2 % on the Low and High treatments, respectively, and the total crude-fibre intake per cow per day was 7·9 and 7·7 lb, respectively.

When the levels of feeding before, during and after the experiment are considered, it can be seen that there was little change in the diet of the High group from the pre-experimental to the experimental period, while the Low group suffered a drop of about 30 % in S.E. In the post-experimental period, however, the High cows suffered a decrease in the amounts of S.E. and D.C.P. allowed while the level of S.E. feeding of the Low group increased appreciably.

Milk yield

The average milk yields for each treatment and group in each period are given in Table 4. In the 11-week period the difference of 4 lb per cow per day in average milk yield between the High and Low treatments was significant ($P < 0{\cdot}01$) and the difference gradually widened throughout the period. With both groups the yield

decreased during the short post-experimental period, but on grass there was a marked recovery in milk yield to 35–36 lb/day. The average total lactation yields for the cows on the High treatment were 10974 lb at 3·82 % fat in 293 days and for those on the Low treatment, 10633 lb at 3·86 % in 301 days. The groups produced on average 1074 and 1063 lb more milk than in the previous lactation. The influence of the levels of feeding in mid-lactation on the total lactation yield was therefore negligible.

Table 4. *Feed consumption and animal production in Expt.* 2

	Pre-experimental 3 weeks		Experimental 11 weeks		Post-experimental 2 weeks		At grazing 3 weeks (after 2 changeover weeks)	
Treatment	High	Low	High	Low	High	Low	High	Low
Feed consumption, lb dry matter per cow per day								
Kale	10·2	9·5	—	—	—	—	—	—
Mangolds	—	—	—	—	4·6	5·1	—	—
Silage	—	—	13·6	14·9	6·5	6·4	—	—
Hay	9·1	9·1	6·6	7·9	9·0	9·2	—	—
Concentrates	14·5	13·2	15·9	4·5	8·9	8·2	—	—
Total	33·8	31·8	36·1	27·3	29·0	28·9	—	—
D.C.P.	3·74	3·51	3·60	3·18	2·76	2·72	—	—
S.E.	21·3	19·8	20·6	13·5	15·7	15·5	—	—
% Woodman's standards								
D.C.P.	110	105	107	105	92	100	—	—
S.E.	119	113	116	83	97	104	—	—
Animal production, lb per cow								
Milk/day	44·6	44·7	41·5	37·5	36·0	32·9	35·6	35·3
Live weight	1152	1073	1208	1086	1171	1049	1101	991
Live-weight gain/day	+0·4	+0·5	+0·4	−0·04	*	*	+1·0	+1·3
Feed efficiency. Total nutrients including maintenance, lb								
D.C.P./gal	0·84	0·79	0·87	0·85	0·77	0·83	—	—
S.E./gal	4·78	4·43	4·96	3·60	4·41	4·39	—	—
S.E./gal (adjusted)	4·39	3·98	4·53	3·64	*	*	—	—
Production nutrients excluding maintenance, lb								
D.C.P./gal	0·68	0·64	0·70	0·67	0·57	0·63	—	—
S.E./gal	3·18	3·04	3·27	1·92	2·47	2·79	—	—
S.E./gal (adjusted)	2·92	2·73	2·98	1·94	*	*	—	—

* Not determined since live-weight change unreliable in short period.

Live weight

The two groups differed in live weight, the High group being on average 79 lb lower in the pre-experimental period. Within each treatment, however, both groups showed similar live-weight changes, being on average heavier in the experimental period than before the experiment, and lighter in the post-experimental and grazing periods. However, when the weights in the pre-experimental and grazing periods were considered the cows in the High group were 81 lb heavier and those in the Low group 54 lb heavier in the experimental period. This difference is in general agreement with linear regressions based on the first 9 weeks which showed an average gain

within the experimental period of 0·4 lb per cow per day on the High treatment and no change on the Low treatment. The major changes from period to period are mainly attributed to differences in gut fill, the substantial increase in weight at the beginning of the experimental period, when all cows changed to a silage and hay diet, being particularly noticeable. Both groups gained in weight on grazing.

Efficiency of feed conversion

Both the gross and net efficiency with which the energy feed was utilized in terms of S.E. per gallon are included in Table 4. Both estimates indicate that the low level of concentrate feeding resulted in a much lower apparent requirement of S.E. per gallon. In contrast to Expt. 1 the D.C.P. allowance exceeded normal standards on both treatments.

Milk composition

The mean composition of the milk from each group of cows is given in Table 5. The differences between the means in the experimental period were not significant but when allowance was made for the differences in group performance in the pre-experimental and grazing periods some of the treatment effects were significant. Calculated on the individual cow results the mean differences in favour of the High treatment were $0{\cdot}20 \pm 0{\cdot}31$ % for fat, $0{\cdot}17 \pm 0{\cdot}04$ % for S.N.F., $0{\cdot}13 \pm 0{\cdot}04$ % for crude protein and $0{\cdot}02 \pm 0{\cdot}02$ % for lactose.

Table 5. *Milk composition* (%) *in Expt.* 2

	Pre-experimental 3 weeks		Experimental 11 weeks		Post-experimental 2 weeks		At grazing for 3 weeks (after 2 changeover weeks)	
	High	Low	High	Low	High	Low	High	Low
Fat	3·51	3·66	3·88	3·82	3·95	3·89	3·83	4·03
S.N.F.	8·71	8·76	8·62	8·55	8·43	8·56	8·58	8·78
Total protein	3·22	3·25	3·17	3·11	3·10	3·16	3·26	3·41
Casein	2·52	2·56	2·40	2·38	2·35	2·45	2·41	2·63
Lactose (anhydrous)	4·61	4·64	4·55	4·57	4·47	4·54	4·49	4·57
Ash	0·725	0·713	0·713	0·690	0·699	0·696	0·706	0·706
Calcium	0·118	0·114	0·117	0·114	0·116	0·116	0·114	0·114
Phosphorus	0·097	0·097	0·092	0·087	0·086	0·086	0·091	0·092
Chloride	0·101	0·100	0·099	0·100	0·109	0·108	0·109	0·106

Similar calculations, using the short post-experimental period, gave very similar differences in favour of the High treatment, namely 0·1 % for fat, 0·16 % for S.N.F., 0·11 % for crude protein and 0·03 % for lactose. The grazing results were preferred for comparison because the post-experimental period was short and differences in level of feeding between the groups were recorded in it.

Consideration of the mean results in Table 5 shows that the milk of the High cows declined in S.N.F. and protein contents from the pre-experimental to the experimental period, that this trend continued into the short post-experimental period (when the level of feeding fell) and that there was only a modest rise when the cows were at grass to approximately the level attained in the experimental period. On the other hand, the cows on the Low treatment showed a more marked fall from the pre-

experimental level, maintained S.N.F. and indeed showed an increase in protein content in the post-experimental period (when their level of feeding rose) and showed a marked rise in S.N.F. and protein when at grazing.

During the detailed analysis of the results it was noted that in the High group, five cows showed a marked drop in mean S.N.F. in the experimental period compared with the pre-experimental and grazing periods while the remaining eight cows maintained or increased their level of S.N.F. Compared in the same way cows on the Low treatment behaved more uniformly, all showing a drop in S.N.F. The means for

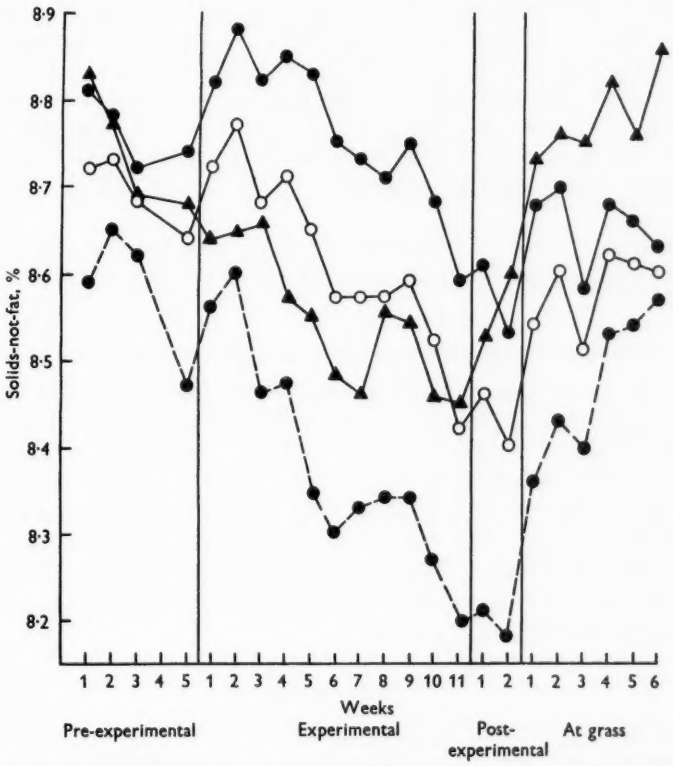

Fig. 1. Mean S.N.F. % per group per week; ○—○ High group; ▲—▲ Low group; ●—● eight cows in High group; ●– –● five cows in High group.

No samples taken in pre-experimental week 4 and data from pre-experimental week 5 not included in Table 5.

the pre-experimental, experimental, post-experimental and grass periods were for the eight cows 8·80, 8·76, 8·57 and 8·64% S.N.F., respectively, and for the remaining five cows of the High group, 8·62, 8·38, 8·20 and 8·49% S.N.F., respectively. The comparable means for crude protein were for the eight cows 3·21, 3·19, 3·10 and 3·23% and for the five cows 3·33, 3·12, 3·09 and 3·29%; for lactose the means were 4·70, 4·67, 4·60 and 4·59% for the eight cows and 4·48, 4·36, 4·25 and 4·33% for the group of five cows.

The changes in mineral content from period to period were small and in this respect the experimental treatments had no significant effect. However, within the High group the milk of the eight cows was lower in ash and chloride contents (0·705 and 0·092 %, respectively) than the milk of the five cows (0·756 and 0·106 %, respectively).

The mean weekly S.N.F. contents are shown for the High and Low groups and for the subgroups of eight and five cows within the High group in Fig. 1. Within the 11-week experimental period S.N.F. contents showed a general decline, although there were week-to-week fluctuations presumably associated with variations in the quality of the hay and silage. Within the 11-week period the regression of S.N.F. % on time was $-0{\cdot}028 \pm 0{\cdot}0012$ % for the High group and $-0{\cdot}020 \pm 0{\cdot}0014$ % for the Low group. This difference in slope was not significant. Within the High group the regression coefficients were $-0{\cdot}023 \pm 0{\cdot}0015$ % for the eight cows and $-0{\cdot}034 \pm 0{\cdot}005$ % for the five cows. The difference in slope was not significant, but the difference between the Low group and the group of five High cows was just significant ($P < 0{\cdot}05$). The marked decrease in S.N.F. for the five cows between weeks 2 and 6 was associated with a decrease of about 0·2 % in protein and of about 0·1 % in lactose.

These declines in S.N.F. % took place although, with the probable exception of week 11, the feeding relative to milk yield was maintained at a constant level on both treatments.

Influence on profitability of milk production in both experiments

Calculations of feed costs and milk returns at 1958 prices (milk 4·2*d.* per lb; feeds per lb dry matter, hay 1*d.*, silage 1·5*d.*, concentrates High 3*d.*, Low 4·5*d.*) showed in Expt. 1 the following margins over feed cost per cow per day during the winter experimental period: treatment A, 86*d.*; B, 83*d.*; C, 84*d.* and D, 80*d.*; and for Expt. 2: High treatment, 100*d.*; Low treatment, 107*d.* These margins, particularly those of Expt. 2, based on a long period, indicate that some increase in profitability may arise from the substitution of cheap fodders for concentrates. The influence of the treatments on the stock-carrying capacity of the farm and on the composition of the milk must, however, also be borne in mind.

DISCUSSION

The experiments described in this paper were carried out to study the effect of plane of nutrition as influenced by the provision of varying allowances of concentrates in addition to silage and hay on the appetite of milking cows and on the quantity and composition of the milk produced. Two different designs were adopted to compare results from the Latin square type of experiment with results from group-feeding experiments, where conditions are more applicable to practical feeding of a dairy herd during the winter period.

In fact a difference in level of feeding as wide as that between treatments A and D in Expt. 1 was not achieved in Expt. 2. The methods of estimating the feeding value of silage and of estimating the level of feeding are both liable to error, and since the cows on the Low treatment were able to maintain weight throughout the 11-week experimental period, it is probable that the silage was in fact of a higher feeding value than estimated. Moreover, the low level of protein feeding which might have con-

tributed to a lowering of yield and S.N.F. content in Expt. 1 (Rowland, 1946) was avoided in Expt. 2. For convenience in the following discussion the results for the mean of treatments A and B in Expt. 1 have been compared with those for the Low treatment in Expt. 2, and the mean results for treatments C and D in Expt. 1 have been compared with the High treatment in Expt. 2. These comparisons show similar responses in the long-term experiment, particularly in the early stages, and the short-term Latin square experiment. Moreover, in milk composition some cows did not respond to the higher feeding level. If they were excluded the response to high feeding shown by the remaining eight cows agreed closely with that in Expt. 1. It should be noted, however, that the overall responses were low compared with those of some other experiments. The mean marginal response to 1 lb additional S.E. was 0·77 lb milk in Expt. 1 and 0·61 lb in Expt. 2, figures which fall in the lower range of the data reviewed by Burt (1957). The response in milk composition was also low. An increase of 16% (Expt. 1) or 19% (Expt. 2) in the level of S.E. feeding was required in the present experiments to raise the S.N.F. contents by 0·1% compared with 10% (Holmes *et al.* 1957) and 13% (Holmes *et al.* 1956) in previous experiments. It is possible that the presence of cows giving low lactose milk, whose effect on response of milk composition to feeding was noted in Expt. 2, and which occurred to some extent in both experiments, may have contributed to the general low response. These indications that circumstances can arise under which cows show no response in milk composition to improved feeding, agree with practical experience on many farms. This problem and its control require further study.

The results give some guidance on factors governing the substitution of grass feeds for concentrates. From the data obtained it can be calculated that an increase of 1 lb of dry matter from concentrates resulted in a reduction in grass intake ranging from 0·18 to 0·22 lb dry matter. This rate of substitution is much lower than that observed by Holmes *et al.* (1957) and Reid & Holmes (1956). The substituted foods contained approximately the same weight of crude fibre and the total crude-fibre intake on all rations was remarkably constant at 7·6–7·9 lb per cow per day. A supplementary digestibility trial made in conjunction with Expt. 2 showed that the indigestible organic matter in both rations was constant at 9·2 lb/day. This suggests that indigestible organic matter might be a suitable measure of the rate of substitution of different feedingstuffs and that crude fibre might be an approximate indication of it. Axellson (1949) and Nordfeldt, Iwanaga, Morita, Henke & Tom (1950) have made similar observations. The importance of the dry-matter content on the palatability of the silage was also noticeable in the present work. Presumably palatability factors also affect food substitution, for example, differences in fibre content do not adequately explain the observations made on feed substitution in the work reported by Holmes *et al.* (1957).

A feature of interest was the fairly consistent decline in S.N.F. during the experimental period, a decline which would be expected from the results of Waite, White & Robertson (1956) who found that the effect of season from January to March resulted in a decrease in S.N.F. which would be greater than the small increase that would be expected in the present experiment from advance in lactation alone. This is sometimes attributed also to the inclusion of large proportions of silage in the diet, but in Expt. 2, although the Low cows received a high proportion of their diet as silage, they showed

a lower rate of decline in S.N.F. than even the eight cows in the High group. Indeed the increases in S.N.F. shown by the Low group in weeks 8 and 9 may be associated with higher silage quality.

The feature of major practical importance from the present results is the finding that despite a low level of concentrate intake in the Low treatment of Expt. 2, the cows in this experiment on average derived their requirements for maintenance plus 2½ gal of milk from hay and silage over a period of 11 weeks; they completed lactations which did not differ materially from those of comparable cows fed much more liberally, although they produced during the winter period milk of relatively low compositional quality. As has been indicated, such results are of economic importance.

The authors are indebted to the Principal and Governors of Wye College and to the Milk Marketing Board of England and Wales for providing facilities for this work, to Mr P. H. Keen, farm manager, and Mr C. Boynton, herdsman, for active co-operation in running the experiments, and to the laboratory and clerical staff who assisted with the analyses and computations.

REFERENCES

AXELLSON, J. (1949). *Proc. 5th Int. Grassl. Cong.* Netherland, p. 266.
BRITISH STANDARDS INSTITUTION (1951). B.S. 1741. Methods for the chemical analysis of liquid milk.
BRITISH STANDARDS INSTITUTION (1955). B.S. 696. Gerber method for the determination of fat in milk and milk products.
BURT, A. W. A. (1957). *Dairy Sci. Abstr.* **19**, 434.
COCHRAN, W. G., AUTREY, K. M. & CANNON, C. Y. (1941). *J. Dairy Sci.* **24**, 937.
DAVIES, W. L. (1932). *Analyst*, **57**, 79.
DIJKSTRA, N. D. (1954). *Netherlands J. agric. Sci.* **2**, 273.
FERTILIZER AND FEEDING-STUFF REGULATIONS (1955). Pamphlet no. 1673. London: H.M.S.O.
HINTON, C. L. & MACARA, T. (1927). *Analyst*, **52**, 668.
HOLMES, W., WAITE, R., MACLUSKY, D. S. & WATSON, J. N. (1956). *J. Dairy Res.* **23**, 1.
HOLMES, W., REID, D., MACLUSKY, D. S., WAITE, R. & WATSON, J. N. (1957). *J. Dairy Res.* **24**, 1.
LUCAS, H. L. (1943). *J. Dairy Sci.* **26**, 1011.
NORDFELDT, S., IWANAGA, I., MORITA, K., HENKE, L. A. & TOM, K. S. (1950). *J. Dairy Sci.* **33**, 473.
POWELL, F. J. N. (1953). *J. clin. Path.* **6**, 286.
RAGSDALE, A. C., THOMPSON, H. J., WORSTELL, D. M. & BRODY, S. (1950). *Bull.* 460 *Mo. agric. Exp. Sta.*
REID, D. & HOLMES, W. (1956). *J. Dairy Res.* **23**, 159.
ROCKSTEIN, M. & HERRON, P. W. (1951). *Anal. Chem.* **23**, 1500.
ROWLAND, S. J. (1938). *J. Dairy Res.* **9**, 42.
ROWLAND, S. J. (1946). *Dairy Ind.* **11**, 656.
WAITE, R., WHITE, J. C. D. & ROBERTSON, A. (1956). *J. Dairy Res.* **23**, 65.
WATSON, S. J. (1939). *The Science and Practice of Conservation—Grass and Forage Crops, II.* London: Fertilizer and Feedingstuffs Journal.
WOODMAN, H. E. (1954). *Bull. Minist. Agric., Lond.*, no. 48.
YATES, F., BOYD, D. A. & PETTITT, G. H. N. (1942). *J. agric. Sci.* **32**, 428.

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Fishy flavour in dairy products

I. General studies on fishy butterfat

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Summary. A distinct fish-oil or cod-liver-oil flavour appeared after storage in commercial butterfat containing, as an antioxidant, nordihydroguaiaretic acid (NDGA) and citric acid dissolved in propylene glycol. The fishy condition was reproducible in the laboratory by the combined effect of NDGA with either citric or lactic acids. The acids alone in butterfat gave oily and rather less clearly defined fishy flavours after storage.

Fishy flavours in milk and other dairy products have from time to time been attributed to bacterial activity (Corfield, 1955), and fishy taints in milk and cream have been caused under certain conditions by feeding cows on sugar-beet products (Davies, 1936). Characteristically, however, fishiness is a storage defect of acid salted butter. It was common in Australian and New Zealand butter in the early years of this century, but although it has disappeared in these countries with the adoption of cream neutralization to a pH of 6·6 and higher, it is still an important commercial problem in countries where cultured-cream butter is made and stored.

The subject of fishiness in butter has been reviewed in recent years by McDowall (1953) and Demeter (1954). The defect is well known and its association with salted butter made from acid cream was first demonstrated by Rogers in 1909. Early investigators believed that the fishy flavour was due to trimethylamine freed by the oxidative decomposition of lecithin. Following the investigations of van der Waarden (1947) and Storgårds & Hieteranta (1949) this theory has been discredited, and it is now generally agreed that fishiness of stored butter is due to products derived from oxidation of the unsaturated fatty acids in the butterfat or in the butter phospholipids. The specific identities of the products responsible for the defect are not known, but there is evidence in the work of van der Waarden (1947) and Holm, Wode & Thomé (1952) that both fishiness and the associated defect of oiliness in butter are due to the formation, through oxidative mechanisms, of α,β-unsaturated carbonyl compounds. Many of the factors concerned in the development of the flavour are obscure and Munro & Barnicoat (1953) have emphasized this lack of fundamental knowledge by their failure in an attempt to induce fishiness in New Zealand butter.

In 1955 a batch of butterfat, prepared commercially in Australia, after holding for

several weeks at room temperature, developed a distinct off-flavour resembling fish-oil. The defect resulted from a certain treatment in processing and was found to be reproducible. In view of the fundamental importance of the fishy defect and the large amount of spoiled butterfat available, a study of the condition was undertaken. Initially the aim of the investigation was the isolation and identification of the compounds responsible for the fish-oil flavour in the butterfat. However, as the flavour under study was found to be similar to, if not identical with, the flavour first observed by Olson & Brown (1942) in washed cream following the addition of ascorbic acid and copper, the scope of the investigation was widened to include the development of fishiness in washed cream and the isolation and identification of the compounds responsible.

The first of this series of papers deals with the occurrence of fishiness in butterfat and the second with the volatile compounds associated with fishiness in this product. The results of the investigation into the fishy flavour of washed cream and the associated volatile compounds will be published later.

EXPERIMENTAL

(1) *The occurrence of fishiness in commercial butterfat*

In the preparation of commercial butterfat in Australia (Coombs, 1956) butter is melted by injection of steam, treated with alkali to dissolve curd and neutralize free fatty acids, and separated in a self-desludging separator. The last traces of water are removed in a falling-film evaporator and the molten fat is shock-cooled to 20 °C before canning. Stainless-steel equipment is used throughout, and butterfat prepared by the process regularly has copper contents less than 0·05 ppm. Phosphorus contents are usually around 10 ppm. indicating a phospholipid content of about 0·025 %.

In 1955, after satisfactory laboratory trials, an antioxidant preparation was added to improve the shelf-life of a number of batches of fat. The preparation consisted of a solution of nordihydroguaiaretic acid (NDGA) and citric acid in propylene glycol; the final concentrations of these ingredients in the fat were NDGA 0·005 %, citric acid 0·01 % and propylene glycol 0·045 %. The fat was distributed in filled vacuum-sealed containers made of unlacquered tinplate.

After about 6 weeks at room temperature the treated fat developed a fishy flavour. The flavour at times, and to some observers, resembled that of sardines or herrings in oil, but it was most generally described as resembling shark-liver- or cod-liver-oil. Some observers considered that there was a 'metallic' flavour element present. The affected fat was held for about 2 years at 2 °C and the fishy flavour was gradually masked by an ill-defined stale oxidized flavour.

When the fishy flavour first became apparent a detailed comparison of the commercial process and the earlier laboratory tests of the antioxidant preparation was made. This revealed that the citric acid in the laboratory tests had been added separately to the fat as a fine powder, whereas in the commercial treatment the citric acid had been completely dissolved in the propylene glycol before being added to the fat.

(2) *Laboratory experiments in the production of fishiness in butterfat*

Some preliminary experiments of limited scope were immediately carried out to determine the cause of the fishy defect. They involved the addition to butterfat of the three constituents NDGA, citric acid and propylene glycol separately and in combination, and a study of the flavour after storage. The results confirmed that the typical fishy flavour developed only as a result of the combined action of NDGA and citric acid, but for the acid to be effective in this way it was necessary to ensure solution in the fat by dissolving it beforehand in the solvent. In all instances where citric acid was added directly to the fat as a powder it remained inert.

When the immediate cause of the fishy flavour was established, a more comprehensive experiment was planned to determine the effects of a number of possible accessory factors, such as copper, air and contact with tinplate, on the development of the defect. The effects were also tested of substituting lactic acid for citric acid and diacetin for propylene glycol as a vehicle for the additives. The substances under test were added to butterfat singly and in various combinations in the following concentrations by weight.

Citric acid	0·003, 0·01 and 0·03 %
Lactic acid	0·014 % (equivalent to 0·01 % citric acid)
NDGA	0·005 %
Copper (as cupric acetate)	0·5 ppm.
Propylene glycol	0·045 %
Diacetin	0·045 %

The fats were prepared commercially from choicest quality butter and were held in 1 lb unlacquered tinplate cans filled under vacuum. Samples were also held in open cans and in glass jars to determine the effects of free access of air and contact with tinplate. The samples were held at room temperature (averaging 20 °C) and at 43 °C. They were tested for flavour at intervals by a panel of qualified graders. The holding periods ranged from 6 weeks at the higher temperature to 3–4 months at room temperature.

RESULTS

The results of the experiment consist of an extensive series of qualitative flavour descriptions and comparisons which are difficult to present adequately in the conventional tabular or graphical form. They have been summarized therefore as follows.

(*a*) *Control butterfat*

Control fat in sealed tins developed only very slight oily off-flavours. In open tins and particularly at 43 °C tallowy flavours developed.

(*b*) *The effect of acid*

Citric acid at the lower concentrations reduced the pro-oxidant effect of added copper. In the absence of copper both citric and lactic acids were pro-oxidant and in sealed cans the resultant flavours were always oily or fish-oily.

(*c*) *The effect of NDGA*

NDGA alone had a perceptible stabilizing effect and the only off-flavours which developed were slight and poorly defined. However, the combined action of NDGA and either citric acid or lactic acid in the fat invariably led to fish-oil flavours, more intense and more clearly defined than with acid alone.

(*d*) *The effect of copper*

Copper alone gave a mixture of tallowy and oily flavours, but with copper and NDGA together the off-flavour was usually oily. Copper greatly speeded up the rate of deterioration and the combination of copper, NDGA and acid led to very strong fish-oil or cod-liver-oil flavours after only 2 weeks' incubation in sealed cans at 43 °C.

(*e*) *The effect of air*

Fish-oil flavour developed more rapidly and with greater intensity in the sealed cans of fat than in the fat exposed to air. In the presence of air off-flavours were usually of the tallowy type, and the fish-oil flavour, when present, was less clearly defined.

(*f*) *The type of solvent*

Oily and fish-oil flavours were equally apparent whether propylene glycol or diacetin was used as a solvent for the additives.

(*g*) *The type of container*

The nature of the container, i.e. glass or tinplate, did not affect the rate of oxidation or the type of off-flavour. Some corrosion of the tinplate occurred with the acid fats, especially at the higher concentrations.

(*h*) *The effect of temperature*

Although deterioration proceeded most rapidly at the higher storage temperature the fish-oil flavour was more clearly defined and recognizable in fat stored at room temperature.

DISCUSSION AND CONCLUSIONS

The conditions favouring the development of fishiness in butterfat in this investigation were the presence of dissolved acid, an antioxidant, a restricted air supply and, to a lesser extent, moderate holding temperatures. Copper was not an essential factor though it intensified the defect when the other factors favoured its development. As the fishy flavour was not influenced by the nature of the solvent this does not appear to have played a significant part, chemically, in its production.

Although it is generally accepted that the flavours of oxidized fat are due to saturated and unsaturated carbonyl compounds of intermediate chain length, little is known of the chemical reactions leading from the initial hydroperoxidation to the formation of particular carbonyl compounds. It is likely, and there is already evidence for this assumption, that each of the major types of off-flavour resulting from oxidation of fat is associated with particular compounds or groups of compounds. The

relative proportions of such groups would be influenced by various factors such as temperature, catalysts, acidity and, no doubt, by the particular chemical environment in which the decomposition of peroxide takes place. This concept of flavour development in oxidizing fat is supported by the observations presented here, in that the factors promoting the development of the fishy defect were typically those which would be expected to influence strongly the chemical reactions following peroxide decomposition.

The nature of this flavour defect, its association with oxidative deterioration and the decisive role of acidity in its formation suggest its close generic association with the fishy, train-oil or cod-liver-oil flavour defect of acid salted butter. Much of the work dealing with the defect in butter has been concerned with the factors in the aqueous phase, principally acidity, salt and heavy metal contamination. Reproduction of an intense and characteristic form of fishiness in dry butterfat, however, suggests that the development of the defect might well be governed by factors operating in the fat phase.

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REFERENCES

Coombs, G. W. (1956). *Dairy Eng.* **73**, 69.
Corfield, E. A. (1955). *Dairy Ind.* **20**, 1035.
Davies, W. L. (1936). *J. Dairy Res.* **7**, 14.
Demeter, K. J. (1954). *Dtsch. LebensmittRdsch.* **50**, 169.
Holm, U., Wode, G. & Thomé, K. E. (1952). *Medd. Statens Mejeriförsök*, no. 37.
McDowall, F. H. (1953). *The Buttermaker's Manual.* Wellington: New Zealand University Press.
Munro, P. & Barnicoat, C. R. (1953). *J. Dairy Res.* **20**, 274.
Olson, F. C. & Brown, W. C. (1942). *J. Dairy Sci.* **25**, 1027.
Rogers, L. A. (1909). *Circ. Bur. Anim. Ind. U.S. Dept. Agric.* 146.
Storgårds, T. & Hieteranta, M. (1949). *Proc. XIIth Int. Dairy Congr.* **2**, 389.
van der Waarden, M. (1947). *Monographs on the Progress of Research in Holland during the war.* 12. *Chemical and Physical investigations on Dairy Products*, by Eilers, H. *et al.* Amsterdam: Elsevier.

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Fishy flavour in dairy products

II. The volatile compounds associated with fishy flavour in butterfat

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SUMMARY. Constituents of a flavour concentrate from fishy butterfat were separated by gas chromatography on silicone oil and 'Carbowax 400' columns. Two fractions with distinct flavours were obtained, the one of oily flavour and containing *n*-hexanal, *n*-heptanal, hex-2-enal and heptan-2-one, the other of metallic flavour, containing a single carbonyl compound present in relatively small amount. It is considered that these compounds with the exception of heptan-2-one are mainly responsible for the characteristic fish-oil flavour.

Other compounds isolated include propanal, *n*-pentanal, *n*-octanal, *n*-nonanal, *n*-decanal, acetone, pentan-2-one, nonan-2-one, undecan-2-one, acraldehyde, pent-2-enal, hept-2-enal, oct-2-enal, non-2-enal, hepta-2,4-dienal, a compound resembling octa-2,4-dienal, a compound with a mushroom flavour, and a compound that formed the 2,4-dinitrophenylosazone of methylglyoxal.

In 1955 a commercial batch of butterfat developed a fish-oil flavour. It had been treated with a solution of nordihydroguaiaretic acid (NDGA) and citric acid in propylene glycol. The preceding paper (Pont, Forss, Dunstone & Gunnis, 1960) gives a detailed account of the occurrence of the fish-oil flavour and experiments on its formation. This paper deals with the recognition of the compounds responsible for the flavour.

METHODS

(1) *Source of fish-oil flavoured butterfat*

The compounds responsible for the fish-oil flavour were first isolated from the commercial butterfat which developed this flavour. Later they were isolated from butterfat in which a fish-oil flavour had been developed in the laboratory by the incorporation, in propylene glycol solution, of citric acid, NDGA and copper.

(2) *Distillation from butterfat*

The method for the distillation of the flavouring compounds was based on the work of Riemenschneider, Herb, Hammaker & Luddy (1944). Steam was passed through a sintered glass disk (No. 2 porosity) into the butterfat at 50 °C and 20 mm mercury pressure. Most of the water was condensed in an ether condenser ('Quickfit', C11/23) cooled with water at 2 °C, and the remainder was collected in two traps filled

with 3 mm glass beads and immersed in a mixture of solid carbon dioxide and ethanol.

Most of the flavouring material distilled in 2 h. When the distillate was shaken with deodorized fat, the flavour was similar to that of the butterfat before distillation. The flavour in the distillate was stable at 2 °C for at least a week.

Three samples of butterfat with no off-flavours were examined in a similar manner. Two contained NDGA in propylene glycol and the other contained no additives.

(3) *Preliminary tests*

Various reagents were added to the distillate and their effect on taste and on the light absorption spectrum observed.

(4) *Methods of isolation of compounds*

The steam distillates were examined by two methods.

Fractionation of the free compounds

The flavour compounds were extracted from the distillate with light petroleum and further examined.

Liquid-adsorption columns of alumina, Celite and silica gel were used to separate the constituents of the light petroleum concentrates. Of these only the Celite column gave useful separation. In general little information was obtained by means of this technique.

Gas chromatographic separation was applied in the following manner. The flavouring compounds present in 500 ml of steam distillate obtained from 2 kg butterfat were extracted with 250 ml light petroleum (b.p. below 40 °C, free from aromatic hydrocarbons and carbonyl compounds). The extract was dried over sodium sulphate at −5 °C for 15 h and concentrated to 1–5 ml in a semimicro fractional distillation apparatus.

This concentrate was introduced into a chromatographic column operating at 45 °C through a 10 ml delivery pipette bent in the form of a U and connected to a by-pass device (Forss & Stark, 1958). Helium was passed through the pipette immersed in a water-bath at 35 °C until the solvent had passed on to the column. The pipette was then heated at 200 °C for 5 min. When all the light petroleum had passed through the apparatus ($\frac{1}{2}$–1 h) the temperature of the chromatographic column was gradually raised to the desired figure, for example, 120 °C in $\frac{1}{2}$ h.

As stationary phases both silicone oil and 'Carbowax 400' gave good fractionation. Fractions from one column were trapped and re-run on the other column for further purification or to obtain other retention times. At first fractions were collected every 5 min and examined for flavour by addition to bland butterfat. It was found that an oily flavour (similar to that of liquid paraffin or engine oil, with a slight flavour of grass) was eluted before a metallic flavour.

Fractionation of derivatives

The 2,4-dinitrophenylhydrazones of compounds in the steam distillate were prepared, fractionated and identified.

In a typical experiment, steam distillation of 6 kg of butterfat gave 1 l. of distillate which was mixed with 500 mg 2,4-dinitrophenylhydrazine contained in 20 ml of 50% H_2SO_4. The mixture was left overnight at room temperature and extracted in a liquid-liquid extractor with carbonyl-free light petroleum (prepared by shaking for 16 h with 2 N-H_2SO_4 saturated with 2,4-dinitrophenylhydrazine, drying and distilling). The aqueous solution was then extracted with carbonyl-free benzene (prepared by the method of Henick, Benca & Mitchell, 1954).

The light petroleum solution was concentrated and the 2,4-dinitrophenylhydrazones fractionated by paper and column chromatography. Earlier work (Forss & Dunstone, 1957) had shown that many 2,4-dinitrophenylhydrazones of saturated aliphatic aldehydes and ketones were chemically altered on silica gel adsorption columns. Partition systems were therefore used. The 2,4-dinitrophenylhydrazones of the carbonyl compounds from the commercially prepared butterfat were fractionated by the sheet-paper chromatographic method of Lynn, Steele & Staple (1956) with light petroleum and paper soaked in 2-phenoxyethanol and this was followed by further fractionation on alumina.

Later, when the fish-oil flavoured butterfat prepared in the laboratory was examined, the 2,4-dinitrophenylhydrazones were fractionally eluted from Celite—nitromethane columns with light petroleum according to Day, Bassette & Keeney (1960) and this was again followed by fractionation on alumina.

(5) *Methods of identification of compounds*

The retention times and the approximate amounts of the separate compounds were determined from the peaks on the gas chromatograms. Fractions were collected for flavour assessment, measurement of ultra-violet and mass spectra, and for conversion to 2,4-dinitrophenylhydrazones.

The 2,4-dinitrophenylhydrazones were identified by measurement of their ultra-violet absorption maxima in ethanol and the spectra of the alkaline ethanolic solutions according to Jones, Holmes & Seligman (1956). Particular attention was paid to the peak in the 520 mμ region which rapidly disappeared with aliphatic saturated aldehydes which are unstable under the alkaline conditions, but persisted with the saturated ketones. Light-absorption measurements were made on a Unicam S.P. 500 spectrophotometer.

The extinctions ($E^{1\%}_{1cm}$) of the isolated and authentic 2,4-dinitrophenylosazones of methylglyoxal in chloroform and the infra-red spectra of Nujol mulls of these two compounds were also measured.

Paper chromatograms were used to compare the movement of the isolated with that of the authentic compound. Three methods were used—that of Klein & de Jong (1956) for the high molecular weight compounds, that of Lynn *et al.* (1956) for the intermediate and that of Meigh (1952) for the low.

Micro-melting points were at first measured on a heated copper block and later with a Kofler-Reichert microfusion apparatus.

To reveal their flavour, the carbonyl compounds were regenerated from their 2,4-dinitrophenylhydrazones with laevulinic acid (Keeney, 1957).

RESULTS

(1) *Preliminary tests*

The fish-oil odour in the steam distillate was destroyed by N-HNO_3, 0·01 N-NaOH, N-NaOH, N-H_2SO_4 saturated with 2,4-dinitrophenylhydrazine, silver oxide, potassium permanganate, semicarbazide and sodium bisulphite. N-HCl and to a lesser extent N-H_2SO_4 partly removed a metallic element from the flavour, leaving it predominantly oily. These results indicated that the tainting compounds were aldehydes.

The steam distillate had three light-absorption maxima in water at 217, 241 and 276 mμ. When the steam distillate was treated with semicarbazide, two peaks in the absorption spectrum were observed at 222 and 291 mμ, corresponding respectively to that of a saturated aldehyde semicarbazone (e.g. *n*-heptanal, 222 mμ) and the 2,4-dienal derivative (e.g. hepta-2,4-dienal, 293 mμ). When the steam distillate was treated with silver oxide, N-HNO_3, semicarbazide, 2,4-dinitrophenylhydrazine and N-NaOH, and the solutions distilled, the distillates showed little absorption between 210 and 310 mμ.

(2) *Characterization of the flavour compounds*

Free compounds

The light petroleum extract of the distillate had an absorption peak at 217 mμ (e.g. hept-2-enal, 217 mμ) and another at 264 mμ (e.g. hexa-2,4-dienal, 262 mμ; Hirayama, 1955). When such an extract was added to a Celite chromatographic column and developed with light petroleum, the compounds with an oily-metallic flavour were eluted first and had a $\lambda_{max.}$ at 218 mμ, while a cardboard-paint flavoured fraction which followed had a small peak at 218 mμ and a large peak at 264 mμ. These two fractions were treated with 2,4-dinitrophenylhydrazine and the 2,4-dinitrophenylhydrazones extracted with light petroleum and fractionated on paper (Meigh, 1952). The positions of the spots were compared with those of *n*-alkanal 2,4-dinitrophenylhydrazones and the compounds were then eluted with ethanol and their $\lambda_{max.}$ values measured. The 2,4-dinitrophenylhydrazones of the oily-metallic flavoured compounds appeared to be saturated or non-conjugated unsaturated compounds while the cardboard-paint flavoured fraction contained conjugated unsaturated carbonyl compounds.

Tables 1 and 2 show the results of the gas chromatographic fractionation of the flavour compounds and their addition to bland butterfat for flavour judgements. On both columns, only two fractions (2 and 4 in Table 1 and 3 and 5 in Table 2) produced a significant flavour when added to the butterfat. When these two fractions were both added the flavour was similar to that of fish-oil flavoured butterfat.

The first fraction described as 'strong oily' contained *n*-hexanal, *n*-heptanal, hex-2-enal and heptan-2-one, while the metallic flavour in the second fraction was caused by a single carbonyl compound which behaved on the two columns in a manner similar to hept-2-enal but remained on the columns a few minutes longer. Measurement of retention times of standard aldehydes and ketones on the two columns showed that the metallic-flavoured compound was not a *n*-alkanal, a 2-methyl-*n*-alkanal or a *n*-alkan-2-one.

From the areas of the peaks on the gas chromatograms and their analysis through their 2,4-dinitrophenylhydrazones the amounts of all the carbonyl compounds isolated were determined (see Table 3).

When a mixture of *n*-hexanal, *n*-heptanal and hex-2-enal in the ratio isolated was added to butterfat, the flavour was described as typically oily.

Table 1. *Gas chromatography of the fish-oil flavour compounds on silicone oil*

Fraction	Compounds isolated in fraction	Flavour of fraction in butterfat
1	*n*-Pentanal, pentan-2-one, pent-2-enal	—
2	*n*-Hexanal, hex-2-enal, *n*-heptanal, heptan-2-one	Strong oily
3	Hept-2-enal, 'mushroom compound'	Mushroom
4	'Metallic compound'	Strong metallic
5	Trace 'metallic compound', *n*-octanal, hepta-2,4-dienal	Slight metallic, cardboard
6	Oct-2-enal, *n*-nonanal, nonan-2-one, octa-2,4-dienal-like compound	Slight tallowy
7	Non-2-enal	Cucumber
8	*n*-Decanal, undecan-2-one	—

Table 2. *Gas chromatography of the fish-oil flavour compounds on 'Carbowax* 400'

Fraction	Compounds isolated in fraction	Flavour of fraction in butterfat
1	Propanal	—
2	*n*-Pentanal, pentan-2-one	—
3	*n*-Hexanal, pent-2-enal, *n*-heptanal, heptan-2-one, hex-2-enal	Strong oily
4	*n*-Octanal, 'mushroom compound', hept-2-enal	Mushroom
5	'Metallic compound', *n*-nonanal, nonan-2-one	Strong metallic
6	Oct-2-enal, hepta-2,4-dienal, *n*-decanal, non-2-enal, undecan-2-one	Cucumber, tallowy

2,4-*Dinitrophenylhydrazones*

Regeneration of the carbonyl compounds from 2,4-dinitrophenylhydrazones showed that the compounds responsible for the oily flavour were represented by that fraction of the 2,4-dinitrophenylhydrazones soluble in light petroleum and with a $\lambda_{max.}$ in ethanol of 358 mμ and R_F values from *n*-hexanal to *n*-heptanal.

The data on the 2,4-dinitrophenylhydrazones of the flavour compounds isolated are compiled in Table 3.

In addition, the 2,4-dinitrophenylosazone of methylglyoxal was isolated from the steam distillate after treatment with 2,4-dinitrophenylhydrazine and extraction with light petroleum and benzene. Both the isolated and authentic derivatives had a $\lambda_{max.}$ in $CHCl_3$ of 396 mμ ($E^{1\%}_{1cm}$ 970); $\lambda_{max.}$ in EtOH-NaOH of 562 mμ; identical infra-red spectra as Nujol mulls; and melting points at 307–8 °C. The identity of the two osazones did not necessarily mean that methylglyoxal was present in the distillate,

Table 3. *Identification of the carbonyl compounds isolated from fish-oil flavoured butterfat*

	Free compound		2,4-Dinitrophenylhydrazone						
			Spectral properties, mμ						
				EtOH–NaOH					
Compound	Percentage of total compounds isolated	Flavour in butterfat	$\lambda_{max.}$ in ethanol	$\lambda_{max.}$	2nd peak	2nd peak persisted	Synthetic compound with identical movement on paper (1) Klein & de Jong, 1956 (2) Lynn *et al.* 1956 (3) Meigh, 1952	Melting points, °C Alone	Mix
Isolated propanal	?	—	356	433	522	No	Propanal (3)	153–5	153
Synthetic propanal	—	Like acetaldehyde	356	432	515	No	—	153–5	–
Isolated *n*-pentanal	4	—	357	432	517	No	*n*-Pentanal (2)	103–5	102
Synthetic *n*-pentanal	—	Sharp, penetrating	358	432	517	No	—	103–4	–
Isolated *n*-hexanal	25	—	356	432	515	No	*n*-Hexanal (2)	102–5	103
Synthetic *n*-hexanal	—	Oily, green leaves	357	432	515	No	—	104–5	–
Isolated *n*-heptanal	20	—	357	433	515	No	*n*-Heptanal (2)	105–6	104
Synthetic *n*-heptanal	—	Oily, fatty	357	432	517	No	—	104–5	–
Isolated *n*-octanal	1	—	357	432	515	No	*n*-Octanal (1)	99–101	104
Synthetic *n*-octanal	—	Fatty, tallowy	357	432	517	No	—	106–7	–
Isolated *n*-nonanal	5	—	357	433	518	No	*n*-Nonanal (1)	102–3	101
Synthetic *n*-nonanal	—	Fatty, tallowy	357	432	518	No	—	102–3	–
Isolated *n*-decanal	<1	—	358	—	—	—	*n*-Decanal (1)	*	–
Synthetic *n*-decanal	—	Orange oil	358	432	518	No	—	104	–
Isolated acetone	?	—	360	432	530	Yes	Acetone (3)	123–5	124
Synthetic acetone	—	Acetone	360	432	530	Yes	—	125	–
Isolated pentan-2-one	4	—	360	432	532	Yes	Pentan-2-one (2)	144–5	145
Synthetic pentan-2-one	—	Like crushed ants	360	432	532	Yes	—	144–5	–
Isolated heptan-2-one	10	—	360	433	530	Yes	Heptan-2-one (2)	68–9	66
Synthetic heptan 2-one	—	Like crushed ants	360	432	532	Yes	—	67–8	–
Isolated nonan-2-one	1	—	361	430	525	Yes	Nonan-2-one (1)	*	–
Synthetic nonan-2-one	—	Like crushed ants	361	432	530	Yes	—	57	–
Isolated undecan-2-one	<1	—	361	430	513	Yes	Undecan-2-one (1)	*	–
Synthetic undecan-2-one	—	Soapy	361	432	532	Yes	—	63	–
Isolated acraldehyde	?	—	368	455	—	—	Acraldehyde (3)	*	–
Synthetic acraldehyde	—	Acrid, suffocating	367	452	—	—	—	165	–
Isolated pent-2-enal	3	—	373	455	—	—	Pent-2-enal (3)	156–8	157
Synthetic pent-2-enal	—	Paint	374	455	—	—	—	157–9	–
Isolated hex-2-enal	4	—	373	455	—	—	Hex-2-enal (2)	145	145
Synthetic hex-2-enal	—	Green leaves	373	453	—	—	—	145–6	–
Isolated hept-2-enal	3	—	373	453	—	—	Hept-2-enal (2)	129–30	130
Synthetic hept-2-enal	—	Cardboard	373	455	—	—	—	130–2	–
Isolated oct-2-enal	2	—	372	453	—	—	Oct-2-enal (3)	127	127
Synthetic oct-2-enal	—	Cardboard, wood bugs	373	455	—	—	—	126–7	–
Isolated non-2-enal	5	—	373	455	—	—	Non-2-enal (2)	123–4	124
Synthetic non-2-enal	—	Cardboard, cucumber	373	455	—	—	—	123–4	–
Isolated hept-2,4-dienal	8	—	389	479	—	—	Hepta-2,4-dienal (3)	154–6	154
Synthetic hept-2,4-dienal	—	Cardboard, linoleum	389	478	—	—	—	153–5	–
Isolated 'octa-2,4-dienal'	3	—	387	477	—	—	Octa-2,4-dienal (3)	185–6	–
Synthetic octa-2,4-dienal	—	—	389	478	—	—	—	153	–

* Insufficient material available for melting point.

since hydroxypropanone and 2-hydroxypropanal both form the same osazone (Neuberg & Strauss, 1945). However, when fresh steam distillate was treated with 5,6-diaminouracil sulphate according to the method of Sattler & Zerban (1949) an absorption peak was obtained at 328 mμ showing that some methylglyoxal was present.

(3) *Control experiments*

The three samples of butterfat which had not developed an off-flavour were steam distilled, the distillates extracted with light petroleum and the concentrates of the extracts fractionated on silicone oil gas chromatographic columns. The peaks were very small compared with those from butterfat with a fish-oil flavour and when fractions were added to butterfat in the same proportions, only those which corresponded to *n*-hexanal, *n*-heptanal and hept-2-enal to hepta-2,4-dienal gave any flavour, and this was very slight.

DISCUSSION AND CONCLUSIONS

This investigation has shown that the fish-oil flavour which developed in butterfat treated with NDGA and citric acid in propylene glycol contained two flavour components, an oily and a metallic, each attributable to certain organic compounds. These can be separated by gas chromatography. The oily flavour component was itself complex and contained *n*-hexanal, *n*-heptanal and hex-2-enal, while the metallic component contained a single powerful-tainting carbonyl compound which has not yet been identified. It had a retention time on silicone oil and 'Carbowax 400' gas chromatographic columns a few minutes longer than hept-2-enal. Its gas chromatographic behaviour indicated that it was not a normal saturated aldehyde, a *n*-alkan-2-one or a 2-methyl saturated aldehyde but possibly a mono-unsaturated aldehyde; it may be a C_8 α-dicarbonyl compound. Characterization through its mass spectrum and 2,4-dinitrophenylhydrazone has not so far been successful, but it is hoped that the identity of this compound will be reported later.

An interesting conclusion from this work is the importance of the two saturated aliphatic aldehydes *n*-hexanal and *n*-heptanal in the oily flavour. Van der Waarden (1947) in his investigation of train-oil flavour in butter added the following substances to bland butter: the C_{1-8} *n*-alkanals, the C_{3-6} 2-enals, octan-2-one and undecan-2-one, the $C_{4-6,11}$ *n*-acids, undecylenic acid, slightly oxidized linoleic acid and seal oil. He found that only *n*-heptanal and seal oil gave flavours resembling train oil. The compounds were added singly, and any combined effects were therefore missed. However, van der Waarden's later conclusion that the compounds responsible for the train-oil flavour 'probably consist of compounds of unbranched aliphatic and probably unsaturated character, with 4 to 8 carbon atoms, with at least one aldehyde group, in addition to which a ketone or an alcohol group possibly occurs' accords with the results reported in this paper.

Tamsma (1955) in his study of heat- and light-induced oxidized flavour in butterfat found that the flavour was partly due to carbonyl compounds which showed no absorption in the ultra-violet region. They could be separated on Celite chromatographic columns in light petroleum and were recoverable in the first fractions.

Similar oily fractions but containing no heptan-2-one have been isolated from butter with a fish-oil flavour churned from washed cream after treatment with copper and ascorbic acid. The heptan-2-one found in the oily fraction was not an artifact produced by heat treatment of the butterfat during processing (see Patton & Tharp, 1959) as only traces of this ketone were found in control butterfats.

The unsaturated aldehyde, hex-2-enal, appeared to contribute little to the flavour, whereas Holm, Wode & Thomé (1952) reported that there was a direct correlation between oily flavour and the amount of α,β-unsaturated carbonyl compounds.

A carbonyl compound with a mushroom flavour was obtained mixed with hept-2-enal from the gas chromatographic columns, and while not important in the fish-oil flavour, may be associated with the mushroom flavour observed in skim-milk exposed to sunlight (Pont, 1951).

There were also identified sixteen other carbonyl compounds which, although they did not contribute to the fish-oil flavour, have been found important, when present in higher concentrations, in other types of oxidized flavour in butterfat. The compounds responsible for these flavour defects will be described later.

There is increasing evidence that whatever the flavour characteristics of an oxidized fat, there may be found in it much the same carbonyl compounds. Thus Day & Lillard (1960, private communication) isolated the C_{1-10} *n*-alkanals and C_{4-11} alk-2-enals but no alk-2,4-dienals and only trace amounts of methyl-ketones from phospholipid-free butterfat exposed to diffuse light at room temperature. Gaddis, Ellis & Currie (1959) isolated a similar range of carbonyl compounds from oxidized pig fats. It appears that particular types of flavour resulting from oxidation of fat are due to the preponderance of certain of these compounds or groups of compounds.

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REFERENCES

Day, E. A., Bassette, R. & Keeney, M. (1960). *J. Dairy Sci.* **43**, 463.
Forss, D. A. & Dunstone, E. A. (1957). *Aust. J. Chem.* **10**, 506.
Forss, D. A. & Stark, W. (1958). *Proc. R. Aust. chem. Inst.* **25**, 201.
Gaddis, A. M., Ellis, R. & Currie, G. T. (1959). *Food Res.* **24**, 283.
Henick, A. S., Benca, M. F. & Mitchell, J. H. (1954). *J. Amer. Oil Chem. Soc.* **31**, 88.
Hirayama, K. (1955). *J. Amer. chem. Soc.* **77**, 383.
Holm, U., Wode, G. & Thomé, K. E. (1952). *Medd. Statens Mejeriförsök*, no. 37.
Jones, L. A., Holmes, J. C. & Seligman, R. B. (1956). *Analyt. Chem.* **28**, 191.
Keeney, M. (1957). *Analyt. Chem.* **29**, 1489.
Klein, F. & de Jong, K. (1956). *Rec. Trav. chim. Pays-Bas*, **75**, 1285.
Lynn, W. S., Steele, L. A. & Staple, E. (1956). *Analyt. Chem.* **28**, 132.
Meigh, D. F. (1952). *Nature, Lond.*, **170**, 579.

NEUBERG, C. & STRAUSS, E. (1945). *Arch. Biochem. & Biophys.* **7**, 211.
PATTON, S. & THARP, B. W. (1959). *J. Dairy Sci.* **42**, 49.
PONT, E. G. (1951). *Aust. J. appl. Sci.* **2**, 326.
PONT, E. G., FORSS, D. A., DUNSTONE, E. A. & GUNNIS, L. F. (1960). *J. Dairy Res.* **27**, 205.
RIEMENSCHNEIDER, R. W., HERB, S. F., HAMMAKER, E. M. & LUDDY, F. E. (1944). *J. Amer. Oil Chem. Soc.* **21**, 307.
SATTLER, L. & ZERBAN, F. W. (1949). *Industr. Engng Chem.* **41**, 1401.
TAMSMA, A. F. (1955). *J. Dairy Sci.* **38**, 284.
VAN DER WAARDEN, M. (1947). *Monographs on the Progress of Research in Holland during the War. 12. Chemical and Physical Investigations on Dairy Products*, by Eilers, H. *et al.* p. 155. Amsterdam: Elsevier.

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With 3 plates

The growth of aerobic spore-forming bacilli in sterilized milk

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Summary. The sterilization of homogenized milk at 115·5 °C for 15 min in bottles having a partial vacuum in the headspace produced conditions inhibitory to the growth from very small numbers of spores of *Bacillus subtilis*, *B. licheniformis*, *B. cereus* and *B. brevis* when compared with growth in the same milk sterilized in open bottles. *B. circulans* differed from the other strains tested in showing greater inhibition in milk sterilized in open bottles than in milk sterilized under partial vacuum.

The extent of the inhibition became less as the size of the inoculum was increased. It became less also as the temperature of incubation approached the optimum growth temperature of the bacillus, and was influenced by the strain of the bacillus and the source of the milk but not by the degree of heat treatment within the range 107–117·5 °C for 15 min. Inhibition was manifested by a prolongation of the lag phase, and in addition with some strains inhibition of spore germination could be demonstrated.

Spore formation following vegetative growth occurred more readily in milk sterilized in open than in evacuated bottles.

Milks sterilized under partial vacuum frequently failed to show any growth from small inocula in 30 days at 22 °C although growth occurred readily in milk sterilized in open bottles.

When 'sterilized milk' was included in the special designations applicable to milk in England and Wales (The Milk (Special Designation) (Pasteurized and Sterilized Milk) Regulations 1949) and in Scotland (The Milk (Special Designations) (Scotland) Order, 1951) no provision was made for a bacteriological standard for this product. Such a standard is desirable and it is therefore important that as much as possible should be known about the growth of micro-organisms in sterilized milk. The use of the term sterilized milk in the dairy industry does not imply that the milk is necessarily sterile. However, in the present investigation no sample of laboratory sterilized milk contained spores other than those added as the inoculum.

No published data are available as to the levels of bacterial contamination associated with a poor keeping quality in commercial sterilized milk. Burton, Akam, Thiel, Grinsted & Clegg (1953) have shown, however, that commercial batch-sterilized milk normally contains 0 to 5 spores/100 ml. They found spoilage in bottles of sterilized milk from the same batch to occur in an erratic manner. This suggests either an uneven distribution of spores between bottles, or that there is some variable factor

or factors which affect the germination and growth of the surviving spores. In a preliminary report, Taylor (1956) showed that the growth of small numbers of spores of the genus *Bacillus* may be inhibited in milk which was sterilized under conditions that were equivalent to those of good commercial practice. Some of the factors which influenced the growth of bacillus spores in sterilized milk are now considered in more detail.

EXPERIMENTAL

Treatment of milk

In an attempt to eliminate contamination with bacterial spores, milk was initially obtained using a sterile milking machine and aseptic technique, from individual cows which were in mid-lactation and free from mastitis. Later, bulk farm supplies proved satisfactory and the variation in chemical composition of single cow samples was avoided. All the milk was derived from cows of the Ayrshire breed.

Milk heated to 75–80 °C was homogenized at 2500 lb/in^2. The homogenized milk was filled into 8, 16 or 40 oz bottles in quantities to give approximately the same headspace relative to volume as is used in commercial practice (approx. 10 %). The bottles were fitted with a perforated screw cap containing a rubber diaphragm and covered with a glass hood to prevent contamination after sterilization. The caps of bottles which were to be sterilized open to the atmosphere ('open' bottles) were placed at an angle across the necks of the bottles and, where necessary, the glass hoods were prevented by curtain hooks from lying flat on the shoulders of the bottles (Pl. 1). The caps of a set of replicate bottles were closed and, by means of a hypodermic needle inserted through the rubber diaphragm, the bottles were evacuated at the water pump for 30 min at a vacuum equivalent to 25 in. Hg and then sterilized ('evacuated' bottles).

For each sample of milk, 'open' and 'evacuated' bottles were sterilized together in a small autoclave. The temperature of the milk during autoclaving was controlled using a thermistor inserted in a similar bottle placed in the centre of the autoclave and containing an equal volume of water. The bottles were heated to the required temperature, held at the temperature for 15 min and allowed to cool in the autoclave for 20–30 min and then on the bench to room temperature. The caps of the open bottles were then closed.

After sterilization the headspace of the evacuated bottles was filled with either commercial nitrogen containing not more than 0·5 % oxygen (A) or with air (B). Aseptic conditions were maintained, the gas being filtered through cotton wool. The headspace of 'open' bottles was filled with nitrogen after removal of air (C) or the bottles were closed with air in the headspace (D). These treatments represent four possible combinations of environmental conditions which might result under commercial conditions of sterilization: (A) Milk which was sterilized and held under partial vacuum, the bottles remaining sealed during and after sterilization. In the laboratory it was necessary to fill the vacuum with an inert gas to permit inoculation and sampling. (B) Milk which was sterilized in a sealed bottle, the seal of which became ineffective after sterilization and allowed air to enter the headspace. (C) Milk sterilized in capped bottles without establishing a vacuum, the seal later becoming closed. (D) Milk sterilized and stored with air in the headspace.

Inoculation

Since the natural microflora of raw milk cannot be sufficiently controlled, pure cultures of mesophilic spore-forming bacilli were added to sterilized milk. Uninoculated samples of the sterilized milk were shown to be sterile.

The following mesophilic bacilli, identified according to Smith, Gordon & Clark (1952) were used: *Bacillus subtilis* (strains 6, D1/2M), *B. licheniformis* (strain 8), *B. cereus* (strain 201), *B. brevis* (strain 58) and *B. circulans* (strains 152, 154).

Stock suspensions of unwashed spores were prepared from nutrient agar cultures which had been incubated at the optimum growth temperature for the strain. The suspensions in quarter-strength Ringer's solution were run through filter-paper, heated at 80 °C for 10 min, cooled immediately and stored at 4–5 °C until required. A sterile 10 ml glass and metal Record hypodermic syringe containing the required dilution of the stock spore suspension was used to inoculate the bottles of sterilized milk through the rubber diaphragms. At the same time, and using the syringe, eight or more plates were each inoculated with 1 ml of the diluted suspension to determine the number of viable spores present in the inoculum. From this was estimated the number of spores in milk containing less than one spore/ml.

Sampling

A $6 \times \frac{3}{4}$ in. test-tube with a rubber bung fitted with inlet and outlet tubes was used to obtain subsamples of sterilized milk (Pl. 2). A hypodermic needle (protected by a $2 \times \frac{1}{4}$ in. tube during sterilization) was connected to the inlet tube, the outlet tube being plugged with cotton-wool.

Before sampling the milk, the bottles were well shaken, the glass hoods removed and the screw caps swabbed with alcohol. Each bottle was clamped in an inverted position, the hypodermic needle of the sampling tube inserted through the rubber diaphragm and the milk allowed to flow into the tube (Pl. 3). If necessary to encourage the flow of milk, a small amount of air or N_2 (as appropriate) to create a slight positive pressure was introduced aseptically into the bottle through the rubber diaphragm of the cap using a hypodermic needle.

Testing

The numbers of bacteria were estimated in plates prepared with 'Oxoid' Lab-lemco agar to which 1 % soluble starch had been added. For spore counts the milk was heated at 80 °C for 10 min and immediately cooled to below 20 °C before plating. Colonies were picked from the countable plates for identification to check the purity of the cultures.

Uninoculated control bottles were included in all experiments. Inoculated and control bottles were sampled at the same time and the sterility of the controls determined by poured plate and microscopic examination.

For direct microscopic examination smears of the milk were dried at 55 °C and fixed by flooding with alcohol and igniting. The smears were stained by flooding with Loeffler's methylene blue diluted 1:15 with distilled water, immediately washing off with water and drying in air.

For the alcohol test, equal volumes of milk and 80 % (v/v) alcohol were mixed and examined at once for precipitation.

RESULTS

Bottles ($\frac{1}{3}$ pint) sealed with crown caps using a hand-capping machine kindly lent by the Crown Cork Co. Ltd. were used in preliminary work. However, variations in the acid-ferricyanide-reducing substances and in acidity (Higginbottom & Taylor, 1960*a*) were observed between bottles of milk sterilized in the same batch. These variations were accompanied by varying degrees of vacuum in the headspace. Satisfactory replication within batches was obtained by the use of a screw cap fitted with a rubber diaphragm which also permitted samples of the milk to be taken from the bottle aseptically without removing the cap. This technique was used then to determine the effect of vacuum in the headspace of the bottles on the concentration of reducing substances in sterilized milk and on the germination and growth of bacterial spores.

The effect of conditions in the headspace during and after sterilization

Growth rate

The growth of bacilli at 37 °C in 'evacuated' bottles of sterilized milk, incubated with either nitrogen (milk A) or air (milk B) in the headspace, was compared with the growth in milk sterilized in 'open' bottles and incubated with nitrogen (milk C) or air (milk D) in the headspace. Comparison was also made with the growth in sterile pasteurized milk (63 °C for 30 min), but after it had been shown that growth was very similar to that in milk D (Fig. 1*c*, *d*) the use of pasteurized milk was discontinued. The milk in the control bottles was sterile in these and all the experiments reported subsequently.

The growth rate at 37 °C of three species of bacilli in milk given treatments A, B, C and D is shown in Fig. 1 for two representative experiments per strain. With *B. subtilis* 6 in milk from cow 1 (Fig. 1*a*) the difference in growth rate between treatments was not marked, although the viable count fell slightly more rapidly in milks held under nitrogen (A and C) than under air (B and D). This was confirmed with another sample of milk from the same cow. Using the same level of inoculum (equivalent to 0·05 spores/ml) and milk from cow 2 (Fig. 1*b*) growth was much slower in milk A, that is in milk sterilized in 'evacuated' bottles and held under nitrogen, than in milks B, C or D in which the growth rate showed no marked differences between treatments. In milk from both sources, however, growth was initiated most rapidly in milk D and least readily in milk A, i.e. the onset of growth was detected first in milk which had been sterilized in an 'open' bottle and incubated with air in the headspace and last in milk sterilized in 'evacuated' bottles incubated under nitrogen. The rate of growth during the logarithmic phase and the maximum population were similar irrespective of treatment, the difference between treatments being associated with the lag phase.

B. licheniformis 8 gave similar results to *B. subtilis* 6 (Fig. 1*a*) using the same level of inoculum and milk derived from cows 3 and 4. The onset of growth was slower in

milk A than in milks B, C or D with only slight differences in the growth rate between these three treatments.

B. cereus 201 showed a more marked response to the treatment of the milk than

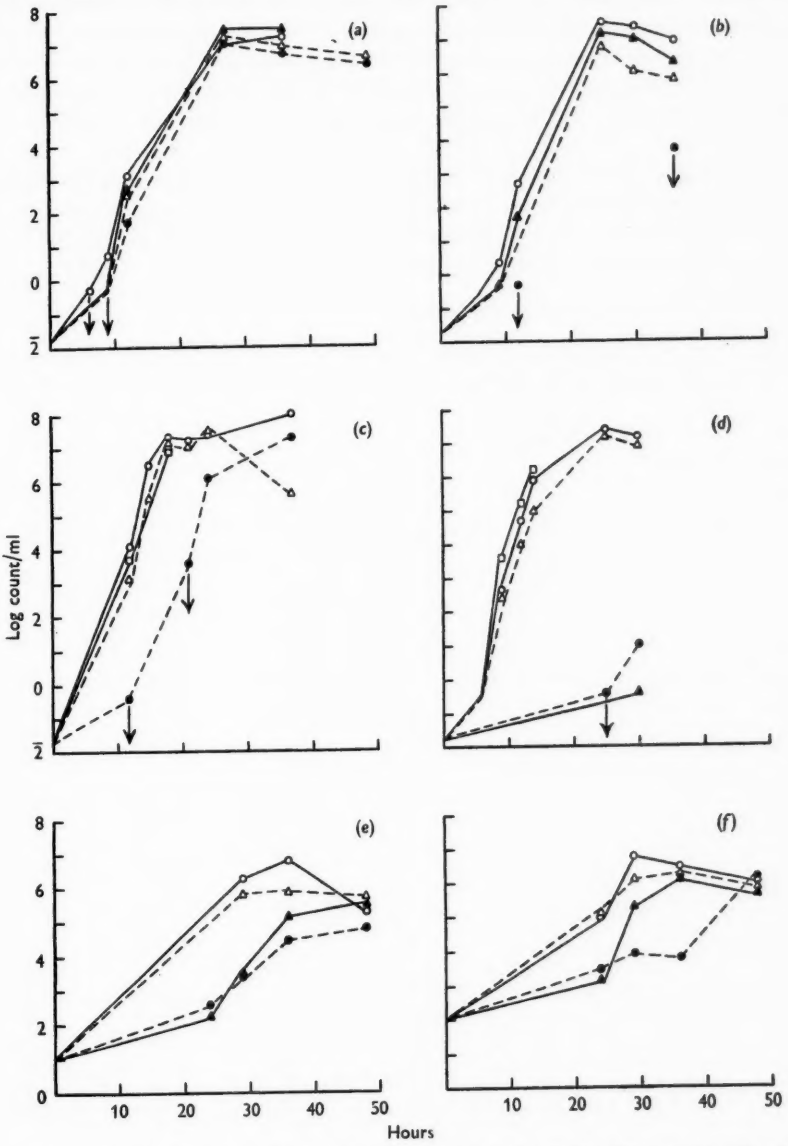

Fig. 1. Growth rate of bacilli at 37 °C in milk treated by methods A, B, C and D and pasteurized E. *B. subtilis* 6, inoculum 0·02 spores/ml, (*a*) milk from cow 1, (*b*) milk from cow 2; *B. cereus* 201, inoculum 0·02 spores/ml, (*c*) and (*d*) milk from cow 1; *B. brevis* 58, inoculum 10 and 100 spores/ml, (*e*) and (*f*) milk from cow 5. Treatments A, ●- - -●; B, ▲—▲; C, △- - -△; D, ○—○; E, □—□; ↓ count below this value.

did *B. subtilis* or *B. licheniformis*. In one experiment (Fig. 1*c*) using milk from cow 2, the growth in milk A was delayed for about 10 h compared with the growth in milks C and D (treatment B was not included). The variation between treatments in the length of the lag phase was even more marked in a second experiment using milk from the same source and a similar level of inoculum (Fig. 1*d*). Growth in milks A and B was retarded for nearly 20 h, i.e. in milks sterilized in 'evacuated' bottles and held under nitrogen or air, compared with growth in milks C and D which were sterilized in 'open' bottles. The rate of growth and the maximum population were similar in milks C and D.

The rate of growth of *B. brevis* 58 was much slower than that of the three strains already mentioned, and inhibition could be shown when the inoculum was increased to 10 or 100 spores/ml. Delay in the onset of growth was shown (Fig. 1*e*, *f*) in milks sterilized in 'evacuated' bottles (milks A and B) when compared with growth in milks C and D.

Table 1. *Spore formation by* Bacillus subtilis 6, B. licheniformis 8, B. cereus 201 *and* B. brevis 58 *in sterilized milk treated by methods A, B, C and D*

Strain	Expt.	Incubation at 37 °C (h)	Spore count/ml of milk A	B	C	D
6	*a*	18	< 0·5	< 0·5	< 0·5	< 0·5
		24	15	3	300	240
		37	> 1000	> 1000	> 1000	> 1000
	b	12	< 0·5	< 0·5	< 0·5	< 0·5
		25	—	49	2	> 10000
		27	—	520	47	—
		30	—	640	117	—
		33	—	760	500	—
		36	< 0·5	1130	860	—
8	*a*	30	< 0·5	< 0·5	< 0·5	< 0·5
		42	< 0·5	< 0·5	0·5	18
		48	22	9	110	700
201	*a*	14	< 0·5	< 0·5	< 0·5	< 0·5
		25	—	—	7	500 app.
		30	< 0·5	< 0·5	28	> 1000
	b	14	< 0·5	< 0·5	< 0·5	—
		26	1	9	2700	—
58	*a*	0	11	13	11·5	10·5
		24	9·5	3·5	3·5	9
		29	4·5	6	3·5	8·5
		36	8	8	3	500 app.
		48	15·5	15	500 app.	> 1000
	b	0	127	133	126	121
		24	55	59	37	40
		29	62	54	34	38
		36	60	41	43	38
		48	74	383	1000 app.	> 1000

Spore formation

Wynne (1952) has noted that it is generally accepted that spore formation is more sensitive to external conditions than spore germination or vegetative growth. Spore counts were made, therefore, during the growth of four strains of bacilli in sterilized

milks given treatments A, B, C and D. As shown in Table 1 spore formation occurred most rapidly in milk D and was slowest in milk A. Spore formation was greater in milk sterilized in 'open' bottles (C and D) than in 'evacuated' bottles (A and B) irrespective of whether there was air or nitrogen in the headspace. Grelet (1952) considered spore formation of *B. megatherium* in a synthetic medium to be an aerobic process but the present results suggest that there are factors more important than the oxygen supply which affect the growth and spore formation of *B. subtilis* 6, *B. licheniformis* 8, *B. cereus* 201 and *B. brevis* 58 in sterilized milk. The results with *B. subtilis* and *B. licheniformis* (Table 1) are of interest in showing inhibition of spore formation in milk A compared with milk D, although there was little difference in the germination of the inoculum and in the rate of vegetative growth in these particular experiments.

The effect of the size of the inoculum

Bulk farm milk was sterilized at 115·5 °C for 15 min under the two treatments A and D, inoculated, and incubated, at 37 °C until the milk became unstable to 80 % alcohol or for 14 days.

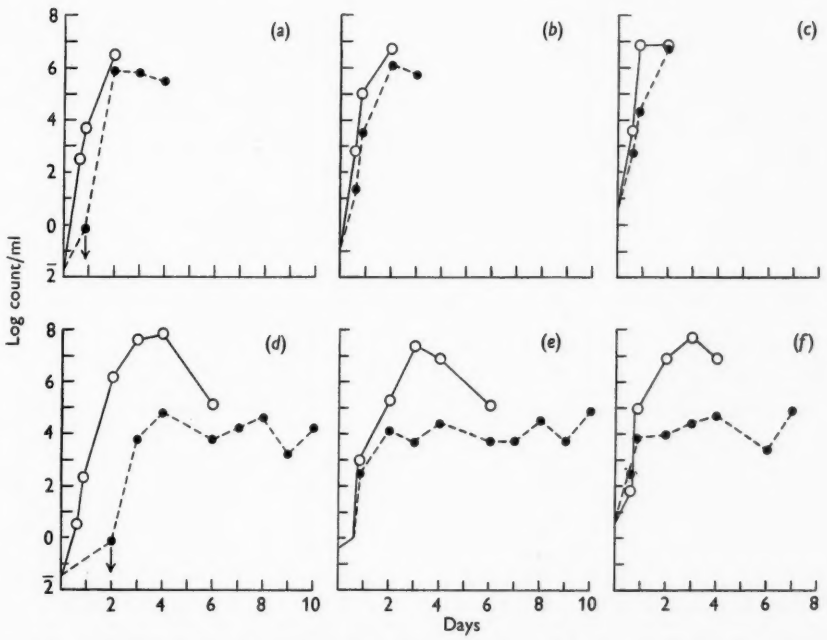

Fig. 2. The effect of size of inoculum on the growth at 37 °C of *B. subtilis* D 1/2 M (inoculum (*a*) 0·02, (*b*) 0·2 and (*c*) 3 spores/ml) and *B. brevis* 58 (inoculum (*d*) 0·04, (*e*) 0·4 and (*f*) 4 spores/ml) in milk given treatments A, ●---● and D, ○—○. ↓ Count below this value.

The lag phase with *B. subtilis* 6 did not always show inhibition in milk A with an inoculum equivalent to 0·05 spores/ml (Fig. 1*a*). When the inoculum was reduced to the equivalent of 0·005 spores/ml there was marked inhibition in milk A. No bacteria were detected in 1 ml milk A after incubation for 14 days at 37 °C, whereas

in milk D growth (25×10^5 bacteria/ml) was well established after 15 h. A second strain of *B. subtilis*, D 1/2 M, showed a slight progressive decrease in the length of the lag phase as the inoculum was increased from 0·02 to 3 spores/ml milk (Fig. 2*a–c*). A more marked decrease in the lag phase in milk B compared with milk D was shown by *B. cereus* 201 when the inoculum was increased from 0·03 to 50 spores/ml. With *B. licheniformis* no delay occurred in the growth in milk A with an inoculum equivalent to 0·02 spores/ml. When the inoculum was reduced to 0·006 spores/ml growth was not detected in milk A during 15 days at 37 °C, but in milk D the bacterial count was 5×10^7/ml in 2 days.

With *B. brevis* 58 using inocula of 0·04, 0·4 and 4 spores/ml (Fig. 2*d–f*), the lag phase in milk A was most pronounced at the lowest level of inoculum. The log-

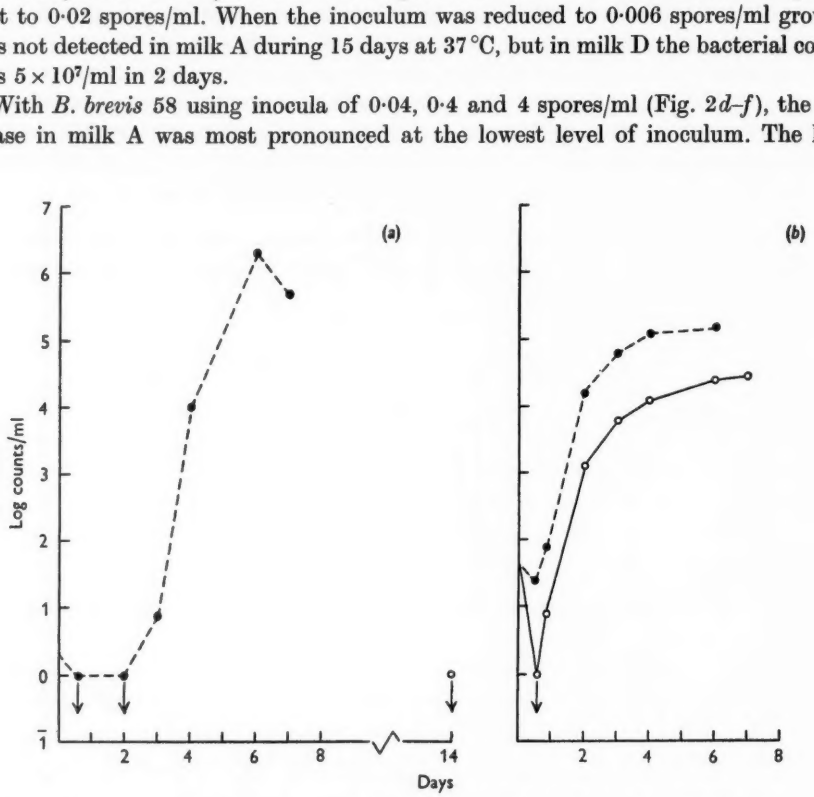

Fig. 3. The effect of size of inoculum on the growth at 37 °C of *B. circulans* 152. Inoculum (*a*) 2, (*b*) 44 spores/ml. Milk given treatments A, ●---● and D, ○—○. ↓ Count below this value.

arithmic growth was independent of the treatment of the milk, but the maximum population with this strain was considerably lower in milk A than in milk D at all three levels of inoculum. The lower maximum population in milk A was maintained for at least 10 days, while in milk D the viable bacteria declined rapidly after attaining the maximum level in 3–4 days.

The behaviour of two strains of *B. circulans* differed from that of the other species of *Bacillus* studied, growth occurring more readily in milk A ('evacuated' bottles under nitrogen) than in milk D ('open' bottles under air). Here also the degree of inhibition was dependent on the size of the inoculum. With an inoculum equivalent to 0·2 spores/ml, growth of *B. circulans* 152 was not detected in either milk A or D.

With 2 spores/ml growth was detected in milk A after 3 days at 37 °C but not in milk D after 14 days (Fig. 3*a*). Growth occurred in both milks with an inoculum of 44 spores/ml but was detected earlier in milk A (Fig. 3*b*). This strain of *B. circulans* showed a fall in the viable count during the early stages of incubation. Similar results were obtained with milk from another farm using inocula of 3, 30 and 300 spores/ml. The inoculum of 30 spores/ml incubated in milk D showed a progressive decrease and no viable cells could be demonstrated between the 5th and 14th days of incubation. With *B. circulans* 154 much smaller inocula were required to demonstrate inhibition of growth. With an inoculum of 0·2 or 9 spores/ml growth occurred in both milks, but was delayed for ½–1 day in milk D compared with milk A. With an inoculum of 0·01 spores/ml growth was obtained in milk A but not in milk D.

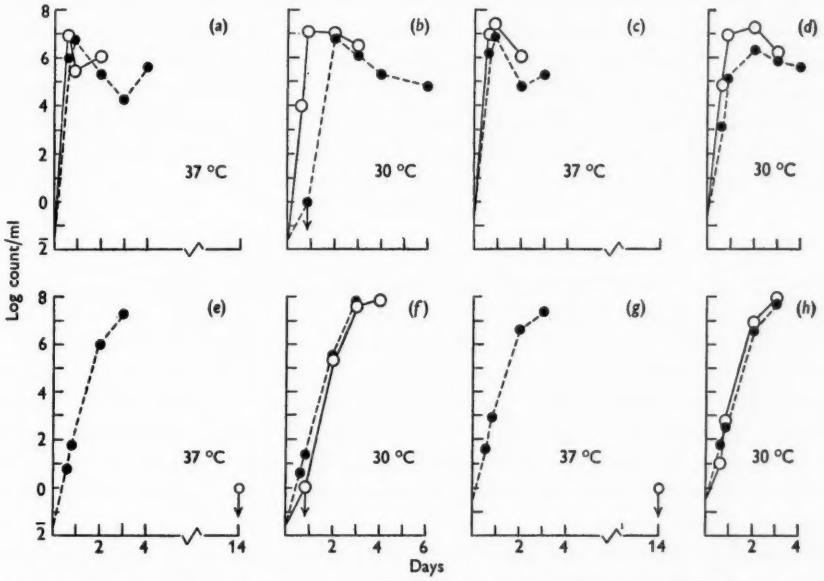

Fig. 4. The effect of size of inoculum on the growth at 37 and 30 °C of *B. subtilis* 6 (*a–d*) and *B. circulans* 154 (*e–h*). Inoculum (*a*), (*b*), 0·03; (*c*), (*d*), 0·2; (*e*), (*f*), 0·02; (*g*), (*h*), 0·3 spores/ml. Milk given treatments A, ●---● and D, ○—○. ↓ Count below this value.

The effect of the size of the inoculum was shown more clearly by incubation at 30 °C instead of 37 °C. Duplicate bottles of milks A and D were incubated at 37 and 30 °C after inoculation with 0·03 and with 0·2 spores of *B. subtilis* 6/ml (Fig. 4*a–d*). At neither level of inoculum was there any sign of inhibition of growth at 37 °C in milk A although with the lower inoculum the milk took 1 day longer to give a positive 80 % alcohol test. At 30 °C the lower inoculum gave a lag period of about 21 h in milk A but no detectable lag in milk D. There was no inhibition with the higher level of inoculum. Similar results were obtained with *B. licheniformis* 8 using milk obtained from another farm. With *B. circulans* 154 (Fig. 4*e–h*) no growth was detected in milk D in 14 days at 37 °C with an inoculum of 0·02 or 0·3 spores/ml. At

30 °C the lag phase was reduced to 21 h in milk D with the lower inoculum but was less marked with 0·3 spores/ml. The degree of inhibition in sterilized milk, therefore, varied not only with the size of the inoculum but with the temperature of incubation. The optimum growth temperature for *B. subtilis* 6 and *B. licheniformis* 8 was 45 °C and for *B. circulans* 30 °C. It seems probable that the inhibition of growth becomes more marked the further the temperature of incubation departs from the optimum growth temperature for the particular strain studied. The effect of temperature will be referred to later.

It has been shown that in milk A, i.e. in milk sterilized under conditions closely resembling those for commercial sterilized milk, small numbers of spores of certain mesophilic bacilli (excluding *B. circulans*) may show delayed germination or growth in the lag phase, and that the level of inoculum at which inhibition may be demonstrated varies with the particular strain and also to some extent with the source of the milk even when bulked farm supplies are used. For instance, delayed growth of *B. subtilis* 6 at 37 °C was not usually shown unless the inoculum was as low as the equivalent of 0·005 spores/ml, but in one batch of milk inhibition was obtained with an inoculum as large as 5000 spores/ml. This variation in sensitivity to milk from different sources was shown by the other strains, but at different levels of inoculum and may be related to variation in the composition of the milk.

Spore germination and vegetative growth

The size of the inoculum required to show an inhibitory effect in sterilized milk varied from about 0·005 spores/ml for *B. subtilis* 6 and *B. licheniformis* 8 to about 50 spores/ml for *B. circulans* 152. This suggested that there might be species differences in the ability of the spores to germinate. The small inocula usually necessary to demonstrate inhibition were unsuitable for the study of germination. However, one particular sample of milk proved to have unusually marked inhibitory properties after treatment A. It was possible with this milk to obtain differences in the growth rate between milks given treatments A and D using large inocula of the order of 5000 spores/ml.

The inhibitory effects discussed above may have been associated with delayed germination of the spores or with slow vegetative growth of the germinated spore. With *B. subtilis* 6 (Fig. 5*a*) over 90 % of the spores had germinated (as determined by loss of heat resistance) after incubation for 9 h at 37 °C in both milks A and D. Although the rate of germination was only slightly slower in milk A than in D there was a marked difference in the total viable count after 9 h, 9×10^3/ml in milk A compared with 5×10^6/ml in milk D. Nearly complete germination of the spores of *B. licheniformis* 8 was obtained at a rate slightly slower than with *B. subtilis* 6 and was slower in milk A than in D. There was no difference in the viable count between milks A and D.

With *B. brevis* 58 (Fig. 5*b*), as with *B. subtilis* 6, rapid germination occurred in both milks A and D. After incubation for 9 h 65 % of the spores had germinated and there was no further increase in germination with continued incubation. Although two-thirds of the spores had germinated in 9 h, vegetative growth was first detected in milk D after 15 h and in milk A after 25 h at 37 °C.

B. circulans 154 showed a different type of response (Fig. 5c) from that given by *B. subtilis* 6 and *B. brevis* 58. Germination of the spores with *B. circulans* 154 was rapid in milk D, nearly 70 % in 3 h, and vegetative growth was detected in 6 h. In milk A vegetative growth was detected after incubation for 9 h, i.e. slightly later than in milk D, although the rate of germination was much slower in milk A. Only about 10 % of the spores had germinated in 15 h but the number of germinated spores increased slowly with further incubation. A type of response similar to that obtained with *B. circulans* 154 was observed with *B. cereus* 201 and *B. circulans* 152. Germination of the spores of strain 152 in milk D, however, was accompanied by a fall in total viable count, and vegetative growth was inhibited in both milks A and D during the period of observation. This suggests that under these conditions auto-sterilization occurs with this strain.

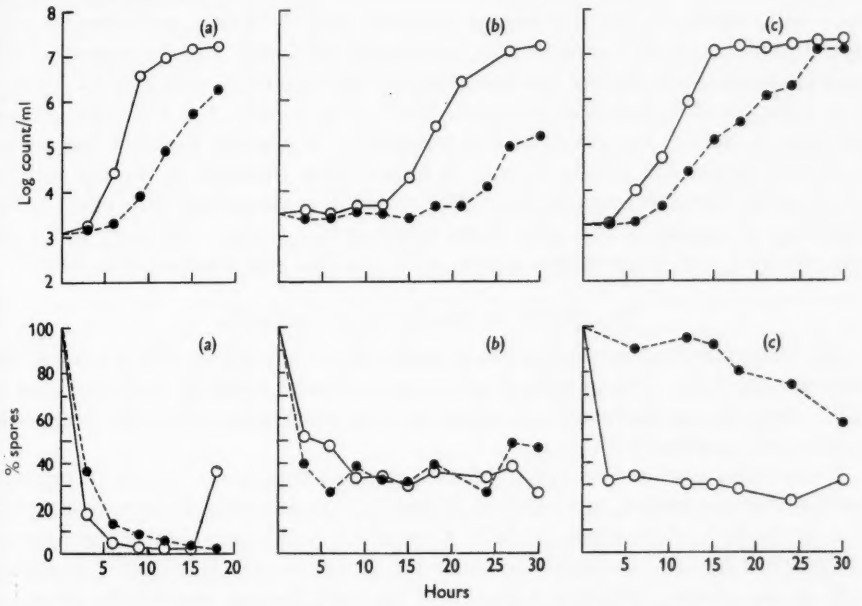

Fig. 5. Spore germination and growth rate at 37 °C of (*a*) *B. subtilis* 6, (*b*) *B. brevis* 58 and (*c*) *B. circulans* 154. Inoculum about 5000 spores/ml. Milk given treatment A, ●---● and D, ○—○.

The results showed that the transition of the cells from the lag to the logarithmic phase, i.e. the vegetative growth, was much more sensitive to the treatment of the milk than the actual germination of the spores with *B. subtilis* 6, *B. licheniformis* 8 and *B. brevis* 58. With these three strains there was a slight delay in spore germination in milk A compared with milk D but this was not so marked as with *B. cereus* 201 and *B. circulans* 152 and 154.

The germination and growth rate of *B. circulans* 154 showed an unexpected reversal of the behaviour with very small inocula where growth was inhibited in milk D, but not with milk A. This suggests that there may be difficulties in applying results obtained with large inocula to the behaviour of very small inocula.

The effect of the degree of heat treatment

No difference was detected in the growth of *B. subtilis* 6 in milk, from the same batch, heated at 107·5, 112·5 or 117·5 °C (4, 8 or 12 lb/in^2 steam pressure) for 15 min. Using an inoculum equivalent to 0·004 spores/ml, growth in milk A was completely inhibited for 14 days at 37 °C irrespective of the temperature of sterilization. In the corresponding milk given treatment D rapid growth occurred at 37 °C. Similar results were obtained with *B. subtilis* D 1/2M using a similar level of inoculum and the same heat treatment, and also with *B. licheniformis* 8 (0·006 spores/ml) in milk heated at 107 and 115·5 °C and with *B. cereus* 201 (0·01 spores/ml) in milk heated at 108·5 and 121 °C. On the other hand *B. brevis* 58 appeared to be more sensitive to the temperature of sterilization of the milk. With milk A a positive 80 % alcohol test was obtained in 11–13 days in milk which had been heated to 107·5 °C, but with the same milk heated to 112·5 °C it was not obtained until 17–18 days, and when the milk was heated at 117·5 °C growth was completely inhibited. In the corresponding milk D, instability to alcohol was observed in 3 days in milk sterilized at 107·5 °C and in 5–7 days in milk heated at 112·5 or 117·5 °C. The growth of *B. circulans* 154 was inhibited in milk D but not in milk A irrespective of whether the milk was heated to 107·5, 115·5 or 117·5 °C for 15 min. It appears that the effect of heating milk for 15 min at temperatures ranging from 107·5 to 121 °C has in general little effect on the inhibitory properties of the milk. Some evidence of increased inhibitory properties was obtained with temperatures above 121 °C but this was not studied in detail.

The effect of the temperature of incubation

The temperature of incubation was normally 37 °C. Growth in milk A at 30 °C was shown to be slower (except with *B. circulans*) and more markedly inhibited than at 37 °C. Since commercial sterilized milk is stored at room temperature, further growth studies were made at 22 °C.

Using milks sterilized at 115·5 °C for 15 min and treatments A and D, replicate bottles were inoculated, incubated at 37 and 22 °C and examined for rate of growth and by the 80 % alcohol test. At 37 °C *B. subtilis* 6 (0·005 spores/ml) showed growth (10^5/ml) in 1 day and instability to alcohol in 2 days in milk D. At 22 °C growth in milk D was slower, 10^2/ml in 2 days, and the milk became unstable to alcohol in 7 days. In milk A no growth or instability to alcohol was detected in 14 days at 37 °C or 32 days at 22 °C. With *B. subtilis* D 1/2M growth in milk D was rapid and the milk gave a positive alcohol test within 3 days at 37 and 22 °C. In milk A growth was not detected in 14 days at 37 °C or 30 days at 22 °C.

With *B. cereus* 201 (0·08 spores/ml) instability to alcohol was delayed by 1 day at 37 °C in milk A compared with milk D. At 22 °C milk D was unstable to alcohol in 7 days but in milk A there was no growth or instability in 32 days.

With *B. brevis* 58 (0·19 spores/ml) growth in milk A was not completely inhibited and was detected in 11–32 days at 22 °C compared with 4 days at 37 °C. Growth in milk D was more rapid than in milk A at both 37 and 22 °C, but showed more difference between 37 °C (10^3/ml in 1 day) and 22 °C (10^2–10^4/ml in 7–11 days) than the other strains of bacilli. Thus the inhibitory effect on the growth of small numbers of spores of certain mesophilic bacilli of milk sterilized under partial vacuum was much

more marked at 22 than at 37 °C. These results emphasize the importance of sterilization of milk in bottles with a vacuum in the headspace in ensuring a long keeping quality in the commercial product.

DISCUSSION

Franklin, Williams & Clegg (1956) and Candy & Nichols (1956) found the *B. subtilis–licheniformis* and *B. circulans* groups to be the commonest species of bacilli causing spoilage in commercial sterilized milk.

The bacilli used in the present investigation were originally isolated from sterilized milk and include representatives of these groups. The strains were chosen also because they varied in oxygen and nutrient requirements and in optimum growth temperatures. *B. subtilis* (6 and D 1/2M) and *B. brevis* are aerobes, and the others facultative anaerobes. The strains of *B. subtilis*, *B. licheniformis* 8 and *B. circulans* 154 were able to utilize ammonia as the sole source of nitrogen and the other strains had more complex nutrient requirements. No connexion was observed between the metabolic requirements and response of these strains to the treatment of the sterilized milk.

Unwashed spore suspensions, stabilized by storage at 5 °C and not subjected to heat-shock immediately before use, were added to the milk after sterilization. To this extent the experimental conditions differed from those in commercial practice, where the surviving spores are subjected to the same heat treatment as the milk. Sufficient control of the small numbers of spores required in the milk was not possible if the spores were added to the milk before heating or if a freshly heated spore suspension was used. Heat activation could not be demonstrated at 37 or 22 °C for any of the strains used but could be shown at 15 °C. Since rapid growth was obtained in sterilized milk under conditions which were not inhibitory, i.e. in milk A with *B. circulans* and milk D with the other strains, the use of stable unheated spore suspensions seems justified. Only one strain, *B. subtilis* D 1/2M, showed delayed germination in the agar medium used for plate counts.

The growth of spores of these mesophilic bacilli with the exception of *B. circulans* has been shown to be retarded in sterilized milk provided the number of spores present was sufficiently small, i.e. within the range found in commercial sterilized milk by Burton *et al.* (1953) and others. Ridgway (1955) was unable to detect any inhibitory effect on spoilage in commercial sterilized milk relative to the degree of vacuum in the headspace within the range '10–70 % of absolute' but he stated that '...there was a slight indication that at a higher vacuum germination and reproduction were retarded, since faults took a little longer to become apparent'. No indication was given of the numbers or species of the bacteria used to inoculate the milk.

In sterilized milk stored under vacuum at 37 and 22 °C, growth was frequently not detected in periods up to 14 and 30 days, respectively, although growth was obtained in the 'open' bottles. This occurred too consistently to be accounted for by failure to add a spore when using very small inocula. It is possible that under these circumstances auto-sterilization occurred or at least that the spores became incapable of germination in the sterilized milk. Curran & Evans (1947) and Wolf & Mahmoud (1957) using relatively large inocula have been able to demonstrate auto-sterilization of spores in nutrient media at temperatures which permitted germination but not

vegetative growth. This has also been demonstrated in the present work with *B. circulans* 152.

An incubation temperature of 22 °C was not below the minimal growth temperature in a non-inhibitory medium for either germination or vegetative growth of the strains used, but it seems that failure to observe growth may be associated in some degree with the loss of the ability of the spores to germinate. A similar explanation may be applied to the observations of Burton *et al.* (1953). They showed that when subjected to batch sterilization at 113 °C for 35 min, the freshly sterilized milk (twelve bottles) contained 0–5 spores/100 ml. After storage at 24 °C for 10 weeks no deterioration in the milk in the remaining 156 bottles was detected judged by physical appearance or taste.

The inhibitory effect observed in the present study was related to the treatment of the milk before and after heating rather than to the degree of heat treatment. The limitation of the oxygen supply was not in itself sufficient to account for the delay in growth. Measurements of pH, titratable acidity and of acid-ferricyanide-reducing substances showed that these properties varied with the degree of heat treatment within the range which failed to affect the growth of the spores. The oxidation-reduction potential was unaffected by the heat treatment used. The relation of these factors to bacterial growth in sterilized milk is discussed in other papers (Higginbottom & Taylor, 1960*a*, *b*).

We thank Mr J. A. McLean of the Physiology Department of the Hannah Institute for making the thermistors, Mr P. B. McCluskey of Lochside Dairy, Ayr, for supplying numerous samples of raw milk and the laboratory staff of the Bacteriology Department, especially Miss M. Price and Miss J. McKaig, for their assistance with the experimental work.

REFERENCES

BURTON, H., AKAM, D. N., THIEL, C. C., GRINSTED, E. & CLEGG, L. F. L. (1953). *J. Soc. Dairy Tech.* **6**, 98.
CANDY, M. R. & NICHOLS, A. A. (1956). *J. Dairy Res.* **23**, 329.
CURRAN, H. R. & EVANS, F. R. (1947). *J. Bact.* **53**, 103.
FRANKLIN, J. G., WILLIAMS, D. J. & CLEGG, L. F. L. (1956). *J. appl. Bact.* **19**, 46.
GRELET, N. (1952). *Ann. Inst. Past.* **82**, 310.
HIGGINBOTTOM, C. & TAYLOR, M. M. (1960*a*). *J. Dairy Res.* **27**, 235.
HIGGINBOTTOM, C. & TAYLOR, M. M. (1960*b*). *J. Dairy Res.* **27**, 245.
RIDGWAY, J. D. (1955). *J. appl. Bact.* **18**, 374.
SMITH, N. R., GORDON, R. E. & CLARK, F. E. (1952). *Agric. Monogr.* no. 16.
TAYLOR, M. M. (1956). *Proc. xivth Int. Dairy Congr.* **1** (2), 487.
THE MILK (SPECIAL DESIGNATIONS) (PASTEURIZED AND STERILIZED MILK) REGULATIONS, 1949. Statutory Instruments no. 1589. Food and Drugs, England.
THE MILK (SPECIAL DESIGNATIONS) (SCOTLAND) ORDER, 1951. Statutory Instruments no. 644. (S. 31). Milk and Dairies, Scotland.
WOLF, J. & MAHMOUD, S. A. Z. (1957). *J. appl. Bact.* **20**, 124.
WYNNE, E. S. (1952). *Bact. Rev.* **16**, 101.

EXPLANATION OF PLATES

Plate 1. Bottles used in the laboratory sterilization of milk. (*a*) 'evacuated' bottle; (*b*) 'open' bottle.

Plate 2. Sampling outfit (see text).

Plate 3. Sampling outfit in operation.

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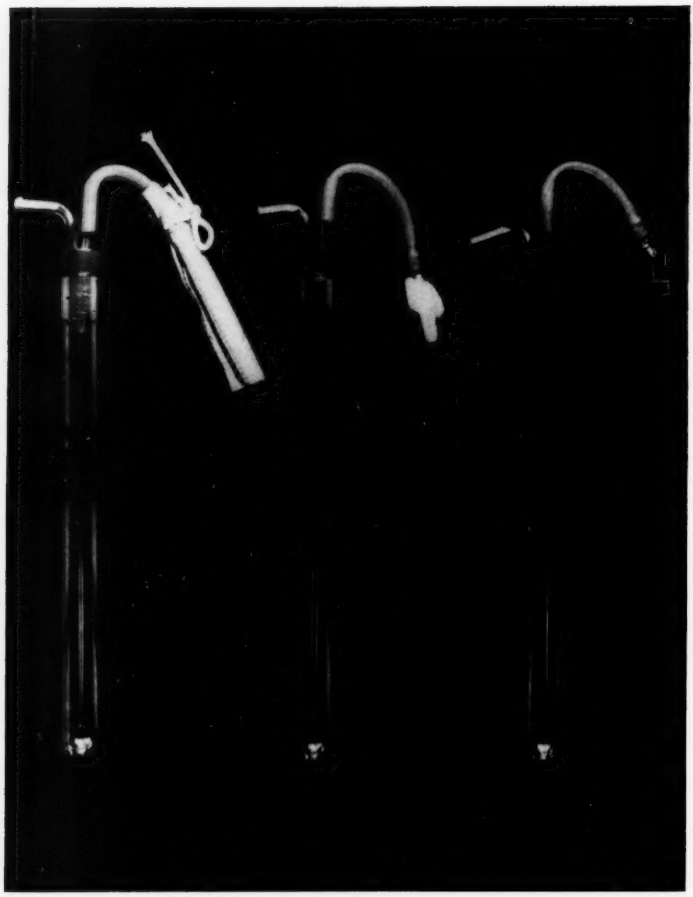

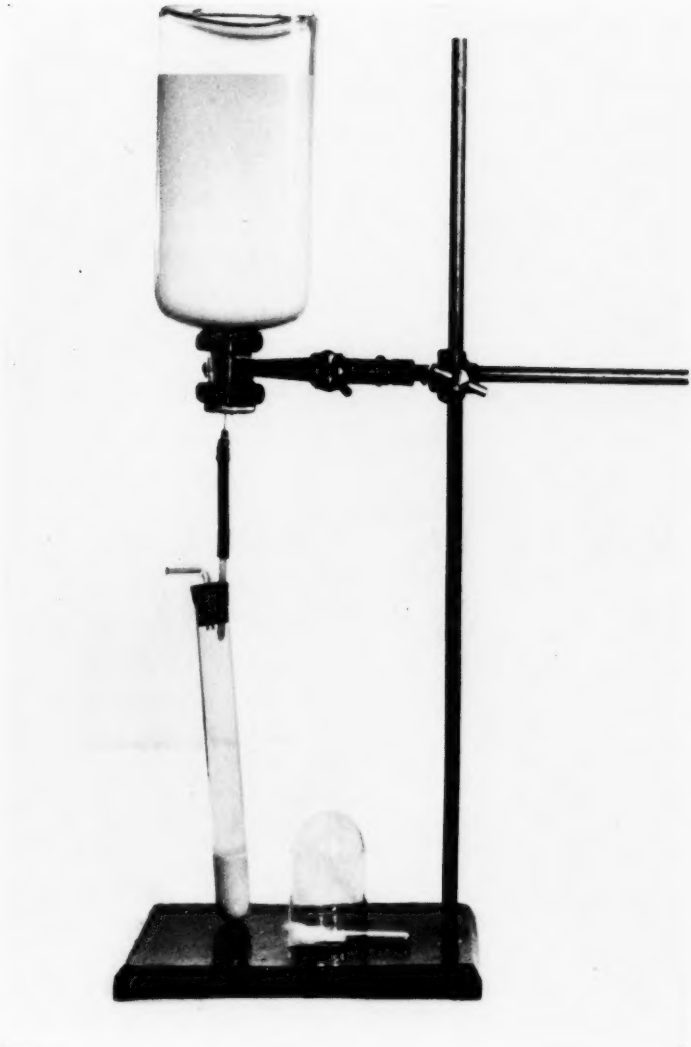

Certain non-biological properties of sterilized milk in relation to the treatment of the milk and the temperature of heating

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Summary. The effect of the temperature of laboratory sterilization of homogenized milk on the titratable acidity, the colour of the milk, the yellowing of the filtrate from the Aschaffenburg turbidity test, the acid-ferricyanide-reducing substances and on pH has been determined. The greater changes in these properties in milk sterilized under reduced oxygen tension compared with milk sterilized in open bottles was shown to be due, in part at least, to the slower rate of cooling in the evacuated bottles. Samples of commercial sterilized milk have also been examined and their properties have been compared with those of the sterilized milk produced in the laboratory.

The ability of spores of the genus *Bacillus* to germinate and multiply in sterilized milk has been shown by Higginbottom & Taylor (1960*a*) to depend in part on the treatment of the milk before and after sterilization. Concurrent with this work, certain non-biological properties of the sterilized milk were examined and considered in relation to the growth in such milks of spores of bacilli. The effect of sterilization under varying conditions on pH, titratable acidity, acid-ferricyanide-reducing substances, the colour of the filtrate in the Aschaffenburg turbidity test and the colour of the milk are reported in this paper. The effect on oxidation-reduction potential will be dealt with in another paper (Higginbottom & Taylor, 1960*b*). These properties have all been followed in the same samples of milk in order to compare the changes caused by the different treatments of the milk and to associate these changes with the rate of bacterial growth.

METHODS

Titratable acidity. 25 ml sterilized milk were titrated with N/9 NaOH to pH 8·60 using a direct reading pH meter in conjunction with a glass electrode and a saturated calomel reference electrode. Results are reported as ml N/9 NaOH/10 ml milk.

pH. A direct reading pH meter was used with a glass electrode.

Turbidity test. The method of Aschaffenburg (1947) was used, the milk (20 ml) and $(NH_4)_2SO_4$ (4 g) being measured accurately. Care was taken to heat the filtrate at 100 °C for exactly 5 min before cooling it in ice-water. These precautions were taken in order to standardize the method for the measurement of the colour of the filtrate.

The yellowing of the filtrate was determined against water in a Spekker absorptiometer using 3 ml filtrate in 1 cm cells and a violet filter (Ilford 601).

Browning of the milk. The colour was measured in red and yellow units (there was no blue component) using a Tintometer in a white-light cabinet. One worker was responsible for all the readings.

Acid-ferricyanide-reducing substances. The method of Chapman & McFarlane (1945) as modified by Crowe, Jenness & Coulter (1948) was used. The milk was diluted 1 in 10 with distilled water and immediately added to the buffered ferricyanide. Mixing by swirling during dilution and addition to the ferricyanide solution was kept to a minimum. Filtration and colour development were done without delay. The colour was measured in a Spekker absorptiometer after development for 10 min and was reported as the drum reading (extinction).

Source and treatment of the milk. Full details are given in the previous paper by Higginbottom & Taylor (1960*a*).

Briefly, the milk was heated to 75–80 °C, homogenized at 2500 lb/in², bottled and heated in an autoclave for 15 min at controlled temperatures ranging from 102·5 to 122 °C. Bottles of milk sterilized with the cap off are referred to as 'open' bottles (milk D); those referred to as 'evacuated' bottles (milk A) were evacuated through the closed cap with a water pump and were kept closed during sterilization.

Bottles of commercial sterilized milk were obtained at weekly intervals from two sources A and B. At A the milk was sterilized by the batch process (AS) and at B by the continuous process (BS). In addition, milk from B was obtained which had been heated in an ultra-high-temperature short time unit (UHT) and also by UHT followed by in-bottle sterilization by the continuous process (UHT-S).

RESULTS

At the start of these investigations the milk was sterilized in ⅓ pint bottles which were sealed with crown caps using a hand-capping machine kindly lent by the Crown Cork Co. Ltd. Subsequent examination of the different bottles within the same batch showed some variation in the results of the tests used, and differences were detected in the degree of vacuum in the headspace of the bottles. Later reproducible results were obtained using screw-cap bottles, and the effect of variation in the degree of vacuum in the headspace was investigated with screw-cap bottles under controlled conditions. The results of the non-biological tests showed that de-aeration of the milk had no effect on the milk itself, but differences in the results of the tests of an order similar to those obtained within batches of crown-capped bottles were found between milks heated in 'open' bottles and those which had been heated with a vacuum in the headspace ('evacuated' bottles).

The effect of heat treatment on the colour, acidity and reducing properties of the milk

Pasteurization and homogenization

The effect of pasteurization for 30 min at 63 °C and of homogenization on the acid-ferricyanide-reducing substances (AFRS), titratable acidity (TA) and pH was determined for the milk of two Ayrshire cows (Table 1). Homogenized milk showed

a slight fall in TA and a small increase in pH compared with the same milk after pasteurization, but any difference between pasteurized and homogenized milk in AFRS was less than the experimental error for the method.

Table 1. *The acid-ferricyanide-reducing substances, titratable acidity, and* pH *of pasteurized and homogenized milk*

	Pasteurized			Homogenized		
Source of milk	AFRS, extinction	TA, ml N/9 NaOH/10 ml milk	pH	AFRS, extinction	TA, ml N/9 NaOH/10 ml milk	pH
Cow 3	0·059	1·397	6·72	0·056	1·309	6·79
Cow 4	0·044	1·456	6·64	0·042	1·425	6·67
	0·072	1·445	6·69	0·071	1·388	6·70

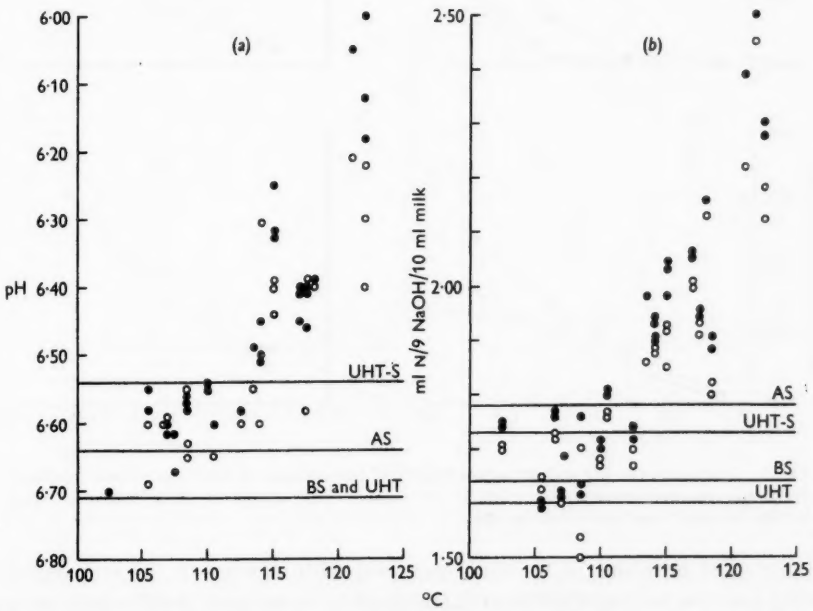

Fig. 1. Changes in the acidity of homogenized milk due to heat treatment for 15 min at temperatures above 100 °C. (*a*) pH; (*b*) titratable acidity. Milk heated in 'open' bottles (o) and 'evacuated' bottles (●). Horizontal lines represent the average values for commercial sterilized milk from plant A (AS) and plant B (UHT, BS and UHT-S).

Laboratory sterilization

pH *and titratable acidity*. The effect of heating for 15 min at temperatures ranging from 102·5 to 122 °C on the pH and TA of the milk is shown in Fig. 1. The pH of the homogenized milk before sterilization was about 6·60–6·75 and after heating at 122 °C it was 6·00–6·30. The pH of the milk heated in 'evacuated' bottles (milk A) was usually 0·1 units lower than that of milk heated in 'open' bottles (milk D) (Fig. 1*a*). The TA increased with the degree of heat treatment, the difference between

milk heated at 102·5 °C and at 122 °C being equivalent to 0·75–0.80 ml N/9 NaOH for 10 ml milk (Fig. 1*b*). The difference between the TA of milks A and D also increased with increase in the temperature of heating. After heating at 120 °C the TA of milk A was greater than that of milk D by the equivalent of 0·15–0·20 ml N/9 NaOH for 10 ml milk.

The increase in TA in both milks A and D after heating in the lower temperature range was not always associated with a corresponding decrease in pH (Fig. 2*a*), although with some milks this buffering effect was not apparent (Fig. 2*b*).

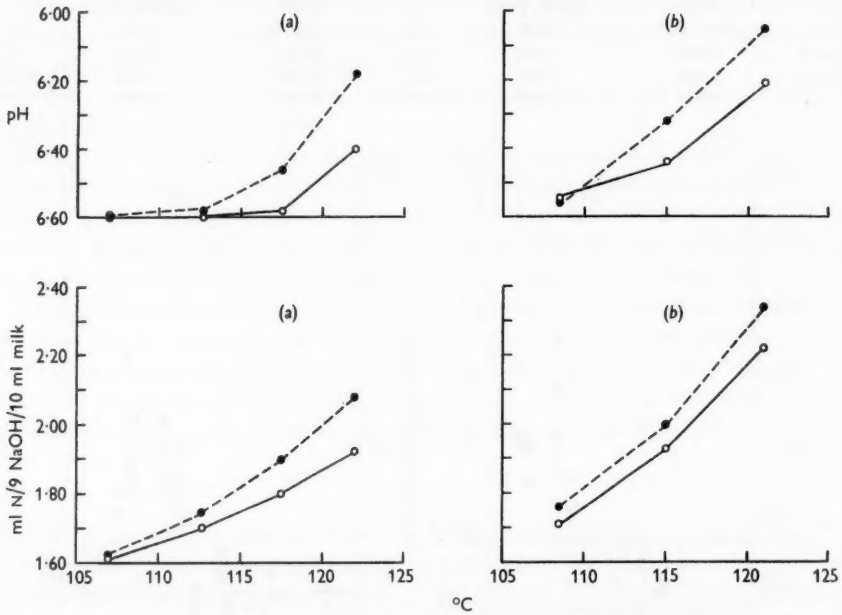

Fig. 2. Changes in the pH and titratable acidity of two samples of milk due to heat treatment for 15 min at temperatures above 100 °C. (*a*) sample 1; (*b*) sample 2. Milk heated in 'open' bottles (○) and 'evacuated' bottles (●).

Colour. The browning of the milk increased with increase in the temperature of heating and was more marked in milk sterilized in 'evacuated' bottles than in 'open' bottles. The results in terms of the red component of the Tintometer reading are shown in Fig. 3*a*.

Yellowing of the filtrate from the turbidity test. All the laboratory-sterilized milks gave a negative Aschaffenburg turbidity test. As previously shown by Clegg & Lomax (1948) and Naudts & de Vleeschauwer (1956), the intensity of the yellow colour of the filtrate was found to increase with the temperature of heating. This increase was greater in milk heated in 'evacuated' bottles than that in 'open' bottles (Fig. 3*b*). The divergence between the results obtained with milk A ('evacuated' bottles) and milk D ('open' bottles) increased with increase in the temperature of heating but was not marked with temperatures below 110 °C.

Acid-ferricyanide-reducing substances. Increase in the temperature of heating from

102·5 to 122 °C was associated with an increase in the AFRS of the milk (Fig. 4). The amount of AFRS was always greater in milk sterilized in 'evacuated' bottles than in milk heated in 'open' bottles.

The results so far described were obtained with milk which had been heated in 220 ml quantities. When larger volumes of milk were sterilized, i.e. 440 ml in 16 oz bottles or 1 l. in 40 oz bottles, greater differences between milk in 'evacuated' and 'open' bottles were obtained in the colour of the milk, acidity and AFRS. It had been assumed that since the 'evacuated' and 'open' bottles were heated together in the same autoclave and placed on the same bench to cool, the total heat treatment

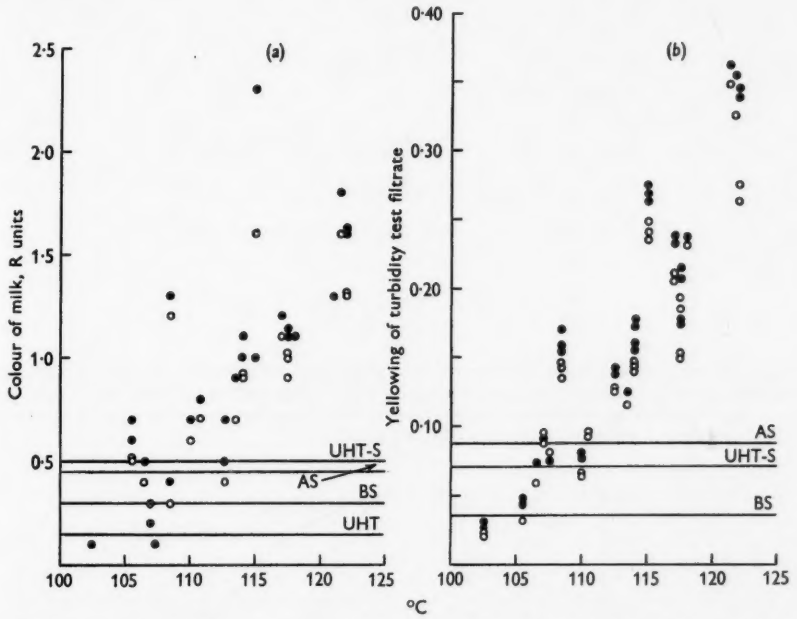

Fig. 3. Changes in the colour of homogenized milk and in the yellowing of the Aschaffenburg turbidity test filtrate due to heat treatment for 15 min at temperatures above 100 °C. (*a*) Colour of the milk, red units (R); (*b*) Yellowing of filtrate as extinctions. Milk heated in 'open' bottles (○) and 'evacuated' bottles (●). Horizontal lines represent the average values for commercial sterilized milk from plant A (AS) and plant B (UHT, BS and UHT-S).

would be the same for all bottles in each batch. Later, in order to verify the assumed identity of treatment, thermistors were placed in both 'evacuated' and 'open' bottles and the temperatures recorded. The results (Fig. 5) showed that although the rate of heating to the required temperature was the same in both bottles, the rate of cooling after the holding period of 15 min was much slower in the 'evacuated' bottles. This difference in the time-temperature relationship between 'evacuated' and 'open' bottles of milk may be sufficient to account at least in part for the more extensive changes observed in milk sterilized in the 'evacuated' than in the 'open' bottles. Heating under vacuum had a definite protecting action on the reducing powers of the milk.

Storage of sterilized milk at 5 °C. After heating milk in 'evacuated' and 'open' bottles at 115·5 °C for 15 min the milk was stored at 5 °C for 7 days. Examination before and after storage showed no change in AFRS, pH or in the yellow colour of

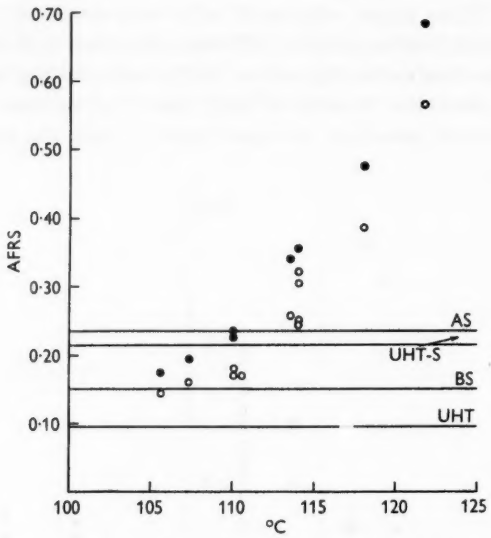

Fig. 4. Changes in the acid-ferricyanide-reducing substances (AFRS) of homogenized milk due to heat treatment for 15 min at temperatures above 100 °C. Milk heated in 'open' bottles (○) and 'evacuated' bottles (●). Horizontal lines represent the average values for commercial sterilized milk from plant A (AS) and plant B (UHT, BS and UHT-S).

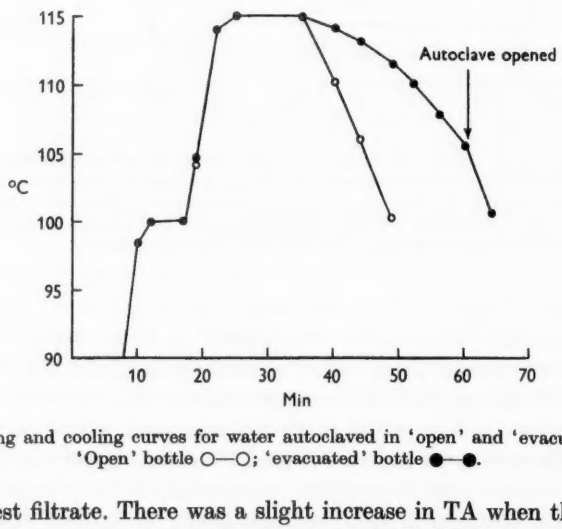

Fig. 5. Heating and cooling curves for water autoclaved in 'open' and 'evacuated' bottles. 'Open' bottle ○—○; 'evacuated' bottle ●—●.

the turbidity test filtrate. There was a slight increase in TA when the value before storage was equivalent to not less than 1·85 ml N/9 NaOH for 10 ml milk. Similar results were obtained for pH, turbidity test filtrate and TA with milk heated at

105·5, 110, 114 and 117·5 °C for 15 min and stored at 5 °C for 41 days. The AFRS showed no change in milk from 'evacuated' bottles, although there was a slight fall in the proportion sensitive to atmospheric oxidation. Milk stored in 'open' bottles showed a small decrease in AFRS. The colour of the milk became whiter during storage and this was most marked in milk which had been heated at 105·5 and 110 °C.

Commercial sterilization. Duplicate bottles of commercial sterilized milk were obtained from two sources A and B at weekly intervals over a period of 6 months (July to December) and examined within 2–5 days of processing. At A the milk was sterilized by the batch process (AS) and at B by the continuous process (BS). In addition, milk from B was obtained which had been heated in an ultra-high-temperature short time unit (UHT) and also by UHT followed by in-bottle sterilization by

Table 2. *Average values for certain non-biological properties of commercial sterilized milk heated by the batch process (AS), the continuous process (BS), the ultra-high-temperature short time method (UHT) and by UHT followed by in-bottle sterilization by BS (UHT-S)*

	Plant A, AS	Plant B		
		UHT	BS	UHT-S
Colour {Red units	0·45	0·15	0·3	0·5
Colour {Yellow units	0·65	0·5	0·65	0·7
Yellowing of turbidity test filtrate, extinction	0·087	—	0·036	0·071
pH	6·64	6·71	6·71	6·54
TA, ml N/9 NaOH/10 ml milk	1·78	1·60	1·64	1·73
AFRS, extinction	0·235	0·097	0·148	0·215
No. of bottles examined	22	7	12	7

the continuous process (UHT-S). The average values for colour, acidity, yellowing of the turbidity test filtrate and for AFRS for the four methods of processing are shown in Table 2. With UHT milk the Aschaffenburg turbidity test was positive and hence did not yield a clear filtrate suitable for measuring the intensity of the yellow colour. With milks from source B the values obtained for colour, acidity and AFRS increased as the amount of processing increased, i.e. UHT < BS < UHT-S. Close agreement was obtained between tests on duplicate samples processed on the same day (Table 3) but some variation was observed from week to week.

For comparison with the laboratory-sterilized milk, average values for the pH, TA, colour of the milk, yellowing of the turbidity test filtrate and for AFRS of the four types of commercial processed milk are given as horizontal lines in Figs. 1, 3 and 4. The values observed for these properties of the commercial sterilized milk (excluding UHT milk) were approximately equivalent to those of milk sterilized in the laboratory for 15 min at 104·5–110 °C.

Effect of aeration. Air bubbled through laboratory-sterilized milk for 30 min caused a decrease in TA and an increase in pH. Usually a decrease in TA equivalent to 0·1 ml N/9 NaOH (10 ml milk) was associated with a change in pH of 0·1 but with some milks the shift in pH was not so large. Aeration also affected the AFRS in milk heated in 'evacuated' bottles. The amount of AFRS was reduced not only by bubbling air through the milk, but also by allowing the milk dilution to stand on the bench for

30 min before examination, or by vigorously shaking to mix the dilution. The reduction in the AFRS value after vigorous shaking during dilution was usually between 10 and 20% of that obtained when the milk dilution was prepared by swirling with the minimum of aeration, there being some variation between milks from different sources. With one milk with an abnormally low TA the average reduction in AFRS due to oxidation during testing was as high as 28%. There was no suggestion in the results that the percentage of oxygen-sensitive AFRS varied with the degree of heat treatment in laboratory-sterilized milk. In the commercial sterilized milk the proportion of oxygen-sensitive AFRS varied from 2·8 to 40·7% of the total AFRS.

Table 3. *Comparison between tests in duplicate bottles of commercial sterilized milk produced on the same day by the batch process (AS), the continuous process (BS), the ultra-high-temperature short time method (UHT) and by UHT followed by in-bottle sterilization by BS (UHT-S)*

Plant	Process	Date	Colour of milk R	Colour of milk Y	Yellowing of turbidity test filtrate, extinction	pH	TA, ml N/9 NaOH/ 10 ml milk	AFRS, extinction
A	AS	11. xi.	0·3	0·5	0·080	6·60	1·756	0·198
			0·4	0·6	0·084	6·61	1·750	0·196
		18. xi.	0·5	0·6	0·081	6·67	1·807	0·290
			0·5	0·7	0·080	6·67	1·800	0·279
B	BS	18. xi.	0·2	0·5	0·028	6·68	1·678	0·158
			0·2	0·5	0·042	6·68	1·682	0·140
	UHT-S	30. ix.	0·5	0·7	0·062	6·64	1·676	0·202
			0·5	0·7	0·068	6·62	1·678	—
	UHT	26. xi.	0·0	0·4	—	6·65	1·580	0·090
			0·2	0·4	—	6·68	1·587	0·087

Fellows (1953) suggested that an acid-ferricyanide test using the method of Crowe *et al.* (1948) was a useful routine method for the detection of variations in the heat treatment of commercial sterilized milk. In the present investigation the estimation of AFRS was made using a 1:10 dilution of the milk, since the 1:2 dilution used by Crowe *et al.* and by Fellows gave a precipitate of prussian blue when used with milk sterilized in the higher temperature range. With milk which gave a suitable colour development with the 1:2 dilution, the values for AFRS were lower than those calculated from the results obtained with the 1:10 dilution, both dilutions being mixed by swirling.

Satisfactory colour development was obtained for all samples of commercial sterilized milk using a 1:10 dilution. When the 1:2 dilution was used, readings could not be obtained (due to precipitation) with batch sterilized milk (AS) irrespective of whether the dilutions were mixed by swirling or by shaking. Milk (BS) sterilized by the continuous process gave satisfactory colour development only if the 1:2 dilution was shaken to mix it. The 1:2 dilution of UHT milk gave satisfactory colour development without aeration but when subsequently sterilized (UHT-S), the aerated 1:2 dilutions gave precipitation of prussian blue which interfered with the readings and the 1:10 dilution had to be used.

DISCUSSION

Changes in pH, TA, AFRS, in the colour of the milk and in the colour of the filtrate from the Aschaffenburg turbidity test have been shown to increase with increase in the temperature of heating of homogenized milk, within the range 102·5–122 °C. Similar changes have been described by other investigators but much of the earlier work referred to skim-milk heated at temperatures below 100 °C and dealt with only one or two of these factors at a time. The simultaneous estimation of these properties in sterilized milk has shown that the amount of change increases with increase in the temperature of heating. The effect is more marked in milk heated with minimum access to air than in milk heated in open bottles, although this difference may in part be explained by the slower rate of cooling of milk in 'evacuated' bottles compared with milk in 'open' bottles. Harland, Coulter & Jenness (1949) found a greater retention of AFRS in milk heated under nitrogen than under air using temperatures below 100 °C.

It has been shown elsewhere (Higginbottom & Taylor, 1960*a*) that the inhibition of the growth of bacterial spores in milk sterilized in 'evacuated' bottles was not appreciably affected by the degree of heat treatment within the range 102·5–120 °C. Further, the size of the inoculum required to demonstrate the inhibition of the growth of small numbers of spores was the same irrespective of the temperature of heating. It therefore seems likely that the heat-induced changes described above, which vary with the degree of heating, have little influence on the growth of bacterial spores in sterilized milk. Alternatively, the effective concentration of the inhibitory factor has been reached at the lowest temperature used and is such that further change at higher temperatures of heating does not increase the inhibitory effect on bacterial spores.

According to The Milk (Special Designation) (Pasteurized and Sterilized Milk) Regulations, 1949 and The Milk (Special Designations) (Scotland) Order, 1951 the only control applied to sterilized milk in Great Britain is that it shall pass the Aschaffenburg turbidity test. While this test is suitable to distinguish between pasteurized milk and milk which has had a more severe heat treatment, it is generally accepted that milk will pass this test after it has been heated at temperatures as low as 85 °C for 30 min as shown by Posthumus (1954). To provide a more satisfactory measure of heat treatment, Clegg & Lomax (1948) suggested the estimation of the yellow colour of the filtrate from the Aschaffenburg turbidity test, Burton (1956) considered the reflectance of the milk and Fellows (1953) proposed the estimation of AFRS. The colour and titratable acidity of milk have been shown here to change slowly on storage at 5 °C and are therefore not suitable except possibly for plant control work. The colour of the turbidity test filtrate may be a useful measure of the degree of heat treatment, but to eliminate personal error the pale yellow colour should be estimated electrometrically. The estimation of AFRS may be a useful method of assessing the degree of heat treatment, but only if the technique is rigidly adhered to and in particular if the amount of exposure to air during sampling and the preparation of the milk dilution is carefully standardized and kept to a minimum. The usefulness of this test depends also on the efficiency of the crown cap in maintaining an air-tight seal on storage.

We thank the Dairy Department of the Birmingham Co-operative Society Ltd. and the Edinburgh and Dumfriesshire Dairy Co. Ltd. who kindly supplied the samples of commercial sterilized milk, Mr J. A. McLean of the Physiology Department of the Hannah Institute for making the thermistors, and the laboratory staff of the Bacteriology Department, especially Miss M. Price and Miss J. McKaig, for their assistance with the experimental work.

REFERENCES

ASCHAFFENBURG, R. (1947). *Mon. Bull. Minist. Hlth Lab. Serv.* p. 159.
BURTON, H. (1956). *Dairy Ind.* **21**, (1), 38; (2), 134.
CHAPMAN, R. A. & MCFARLANE, W. D. (1945). *Canad. J. Res.* **23B**, 91.
CLEGG, L. F. L. & LOMAX, K. L. (1948). *J. Soc. Dairy Tech.* **1**, 245.
CROWE, L. K., JENNESS, R. & COULTER, S. T. (1948). *J. Dairy Sci.* **31**, 595.
FELLOWS, P. V. (1953). *Dairy Ind.* **18**, (4), 309.
HARLAND, H. A., COULTER, S. T. & JENNESS, R. (1949). *J. Dairy Sci.* **32**, 334.
HIGGINBOTTOM, C. & TAYLOR, M. M. (1960*a*). *J. Dairy Res.* **27**, 221.
HIGGINBOTTOM, C. & TAYLOR, M. M. (1960*b*). *J. Dairy Res.* **27**, 245.
NAUDTS, M. & DE VLEESCHAUWER, A. (1956). *Proc. xivth Int. Dairy Congr.* **1**, (2), 185.
POSTHUMUS, G. (1954). *Off. Org. K. ned. Zuivelb.* **46**, (7), 104. Cited in *Dairy Sci. Abstr.* **16**, 418.
THE MILK (SPECIAL DESIGNATION) (PASTEURIZED AND STERILIZED MILK) REGULATIONS, 1949. Statutory Instruments (1949) no. 1589. Food and Drugs, England.
THE MILK (SPECIAL DESIGNATIONS) (SCOTLAND) ORDER, 1951. Statutory Instruments (1951) no. 644 (S. 31). Milk and Dairies, Scotland.

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The oxidation-reduction potential of sterilized milk

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SUMMARY. For milk sterilized under partial vacuum in closed bottles the oxidation-reduction potential measured under nitrogen was markedly lower (−280 to −300 mV) than that of milk heated in bottles open to the air, measurements being made in air (+10 to −30 mV).

The *Eh* (under nitrogen) of commercial sterilized milk and of ultra-high-temperature short-time heated milk was in the range −200 to −300 mV and of the same order as that obtained in the laboratory for deaerated milk sterilized in closed bottles.

Many investigations of the oxidation-reduction potential of milk have been made in connexion with dye reduction or in relation to oxidized flavour. Most of this work has been concerned with raw milk or milk heated to 85–90 °C, but little has been published on the oxidation-reduction potential of milk heated at temperatures over 100 °C. Twigg (1937) found the limiting potential of autoclaved milk in oxygen-free nitrogen at 37 °C to be about −50 mV. Tobler (1955) measured the potential at 50 °C in order to shorten the time required to reach equilibrium. He obtained *Eh* values varying between −80 and −120 mV for milk autoclaved in open vessels, but mentioned that lower values were obtained if the milk were heated in the absence of air.

In the present investigation the effect of the presence or absence of air and of the degree of heat treatment on the oxidation-reduction potential of sterilized milk was studied using methods similar to those of Tobler. Observations were also made on a limited number of samples of commercial sterilized milk.

This work was done in association with the study of bacterial growth in sterilized milk (Higginbottom & Taylor, 1960*a*).

METHODS

Treatment and description of milk

The term 'sterilized' is used throughout to describe either the commercial product or milk treated in the laboratory under similar conditions. In the laboratory, milk was homogenized at 2500 lb/in^2 after preheating it to 80 °C. It was then filled into bottles fitted with screw-caps carrying a rubber diaphragm and the bottles were heated in an autoclave for 15 min at a controlled temperature of 115·5° C except when otherwise stated. Bottles were sterilized while open ('open' bottles), or in

bottles which were closed and evacuated using a water-pump before heating ('evacuated' bottles). A fuller description of the treatment is given by Higginbottom & Taylor (1960*a*).

Measurement of oxidation-reduction potential

The potential was measured using a direct reading pH meter in conjunction with a wick-type saturated KCl-calomel reference electrode and a platinum electrode. The platinum electrode consisted of a bright Pt disk 5 mm in diameter fused to the end of a glass tube. A quinhydrone electrode gave agreement between Pt electrodes of ± 1 mV. When the Pt electrodes were compared in sterilized milk agreement was within ± 5 mV. The cell used, shown in Fig. 1, consisted of a wide-mouthed jar fitted with a rubber bung carrying the two electrodes, gas inlet and outlet tubes, a tube for sampling the milk and when required two more platinum electrodes and a glass electrode.

To determine the *Eh* under anaerobic conditions samples were taken under nitrogen. Commercial nitrogen (stated to contain not more than 0·5 % oxygen) was used, since the experiments were made in conjunction with bacteriological studies in which the presence of traces of oxygen was desirable.

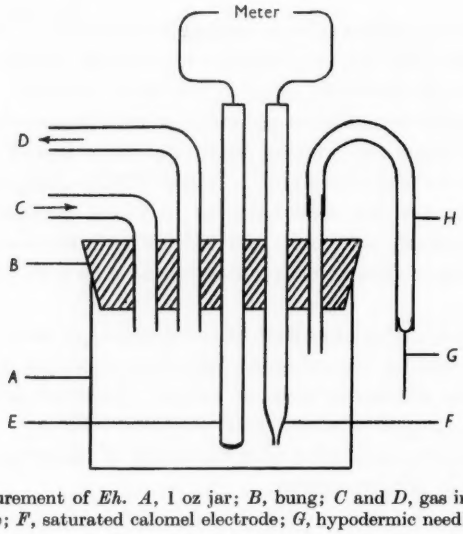

Fig. 1. Cell for measurement of *Eh*. *A*, 1 oz jar; *B*, bung; *C* and *D*, gas inlet and outlet tubes; *E*, platinum electrode; *F*, saturated calomel electrode; *G*, hypodermic needle; *H*, sampling tube.

To sample milk from an 'evacuated' bottle the headspace of the bottle was filled with nitrogen to give a slight positive pressure and the bottle inverted. The measuring cell was flushed with nitrogen. With the nitrogen still flowing through the cell the hypodermic needle was inserted in the rubber diaphragm of the cap of the inverted bottle and the milk allowed to flow into the cell to the required level. When a bottle of commercial sterilized milk was sampled, the crown cap was removed and quickly replaced by a two-holed rubber bung carrying an outlet tube which was connected to a sampling tube attached to the measuring cell, and an inlet tube for nitrogen

(Fig. 2). Nitrogen was passed through the headspace of the bottle and through the measuring cell for 5–10 min before the bottle was tilted to allow the milk to flow into the cell. The cell was held stationary in a water bath at 50 °C and *Eh* readings were taken at intervals of not less than 10 min until a constant value was obtained.

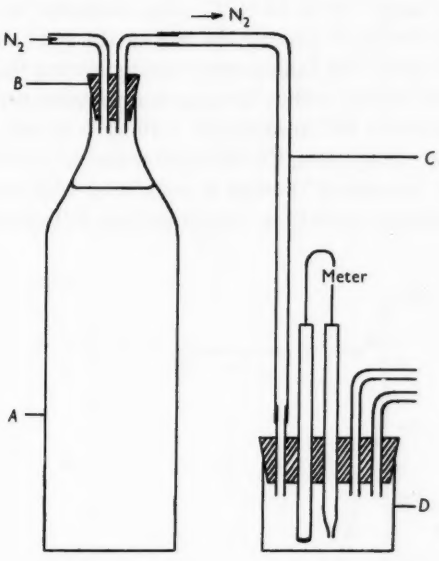

Fig. 2. Method for sampling bottle of commercial sterilized milk. *A*, bottle of commercial sterilized milk; *B*, two-holed bung; *C*, sampling tube; *D*, measuring cell.

RESULTS

Preliminary experiments using 0·002% potassium indigo tri-sulphonate ($Eh'_0 = -61$ mV at pH 6·6 and 30 °C) as an oxidation-reduction indicator showed that the dye was reduced in all but the top 1 cm when the milk was sterilized in 'evacuated' bottles indicating a potential of −120 mV or lower. The same result was obtained when the milk was sterilized for 15 min at 108·5 and 115·5 °C. In addition to the poising action of the dye itself, it was possible to demonstrate a small residual poising capacity in the milk.

To obtain more precise results and to avoid the poising action of the dye all further work was done using the electrometric method.

Laboratory sterilized milk

Since the bacteriological studies with which this work was associated were done mainly in milk which had been heated in 'evacuated' bottles and held under nitrogen (milk A) and in milk which had been heated in 'open' bottles and held in air (milk D) (Higginbottom & Taylor, 1960*a*), oxidation-reduction potential measurements were made under these two sets of conditions. Typical potential-time curves are shown in Fig. 3 for milk from one source. In this example, milk from the 'evacuated' bottle

gave a limiting *Eh* reading of −290 mV and milk from the 'open' bottle showed a slow positive drift rising from +5 to +12 mV in 265 min.

In milk sterilized in 'evacuated' bottles the average value for the *Eh* (under nitrogen) was about −290 mV. Neither the constant *Eh* value (Fig. 4*b*) nor the time required to reach that value (Fig. 5) was affected appreciably by the degree of heat treatment within the range 104·5–117·5 °C. (The variation in the initial readings (Fig. 5), taken within 5 min of placing the cell in the bath, are probably without significance as the potential was falling very rapidly during this period.) However, in milk heated in 'open' bottles within the same temperature range the limiting value for the potential (under air) fell from about +10 to −30 mV with increase in the temperature of heating. It appears, therefore, that the very low potential developed in the milk heated in 'evacuated' bottles is associated with reducing groups which are sensitive to atmospheric oxidation. Higginbottom & Taylor (1960*b*) have shown

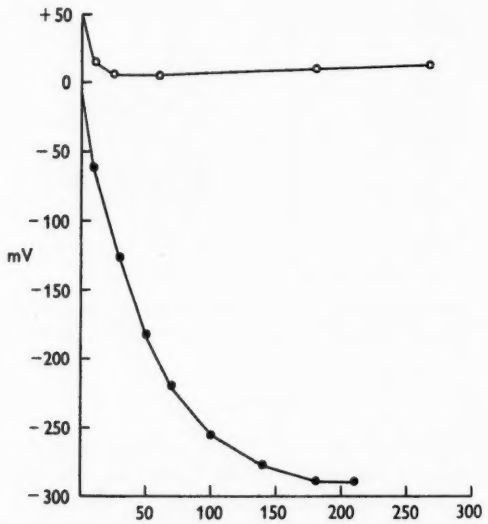

Fig. 3. Potential-time curves for milk heated in an 'evacuated' bottle and measured under nitrogen (●—●) and in an 'open' bottle and measured in air (○—○).

that there is a marked increase in acid-ferricyanide-reducing substances (AFRS) when milk is heated (see also, Fig. 4*a*), but that AFRS sensitive to atmospheric oxidation are obtained only in milk heated in 'evacuated' bottles. If the AFRS which are formed in milk in 'open' bottles during heat treatment, and which are not readily subject to atmospheric oxidation have any influence on the potential of the milk, the change must be slight compared with the potential of milk from 'evacuated' bottles. Obviously, the non-oxygen-sensitive AFRS have a relatively positive oxidation-reduction potential and this will be masked by the more strongly reducing oxygen-sensitive compounds, so that the potential of milk from 'evacuated' bottles does not appear to be affected by the degree of heat treatment. It is possible that

AFRS sensitive to atmospheric oxidation may include the compound or compounds responsible for the low *Eh* of milk sterilized in 'evacuated' bottles.

Like many biological fluids, sterilized milk is a poorly poised system, and it was found that the potential of laboratory sterilized milk could be altered by varying the

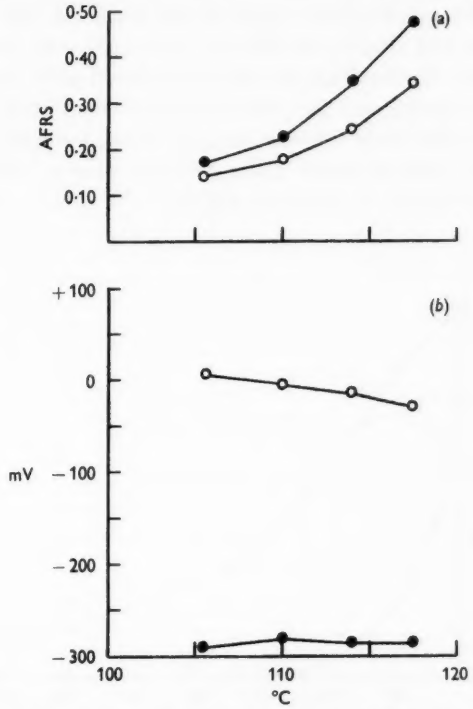

Fig. 4. The relation of acid-ferricyanide-reducing substances (AFRS) and *Eh* of milk in 'open' (○) and 'evacuated' (●) bottles to heat treatment for 15 min at various temperatures. (*a*) AFRS, (*b*) *Eh*; measurements on 'evacuated' bottles made under nitrogen and on 'open' bottles in air.

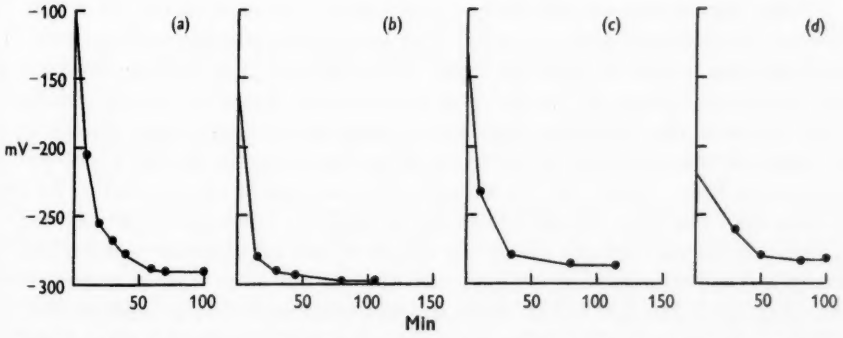

Fig. 5. Potential-time curves for milk sterilized in 'evacuated' bottles for 15 min at different temperatures. (*a*) 104·5°C, (*b*) 110°C, (*c*) 114°C, (*d*) 117·5°C. Measurements made under nitrogen. Values not corrected for pH.

conditions of sterilization or the method of measurement. When atmospheric oxidation was kept to a minimum milk from 'open' bottles could have a very low *Eh* when measured under nitrogen. Thus for one sample the limiting potential was −294 mV in milk from the 'evacuated' bottle and −271 mV for milk from the 'open' bottle (Fig. 6). However, the poising capacity of milk from the 'evacuated' bottle was much greater than for milk from the 'open' bottle, since with the 'evacuated' bottle a constant potential was attained in 120 min compared with 315 min required for milk from the 'open' bottle. This sample of sterilized milk had been closed and stored at 5 °C before testing and the potential-time curve for the milk heated in the 'open' bottle fell steadily from the first reading. When the milk had been in recent contact with the air, measurement under nitrogen gave a 'plateau' effect at the beginning of the curve (Fig. 7*a*, curve 2; Fig. 8).

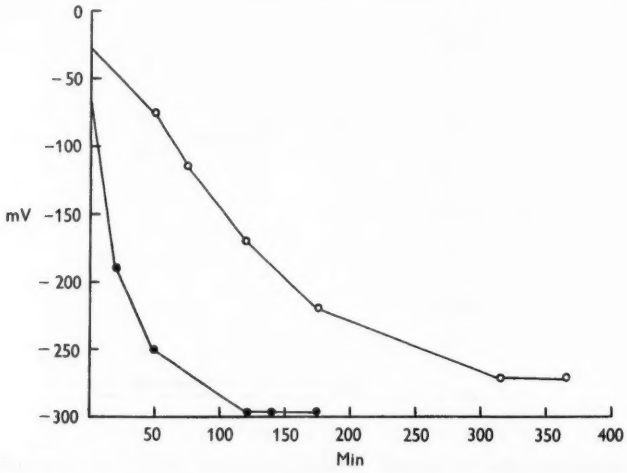

Fig. 6. Potential-time curves for milk heated in an 'evacuated' bottle (●—●) and in an 'open' bottle (○—○) and measured under N_2.

Further information on the effect of atmospheric oxidation on the *Eh* of milk was obtained by determining the potential in air and then in nitrogen and *vice versa*. The potential-time curves for milk from one source showed (Fig. 7) that the *Eh* of the milk heated in 'evacuated' bottles was always lower than that of milk sterilized in 'open' bottles. The milk from the 'open' bottle showed very little change in the *Eh* when air was replaced by nitrogen. (There was actually a very slight positive trend rising from −10 to 0 mV in 300 min. This may not be obvious in Fig. 7*a* owing to the scale.) The *Eh* of the milk from the 'evacuated' bottle gave a steady value of −40 mV in air and did not change for about 40 min after the air was replaced by nitrogen. The potential then fell gradually but had not quite reached a steady value within the next 240 min. When determinations were made first in nitrogen the *Eh* of milk from the 'evacuated' bottle fell rapidly to a constant value of −250 mV in about 150 min and more slowly in milk from the 'open' bottle to reach a steady value of −225 mV in about 250 min. Thus, holding the milks in air for 140 min at 50 °C

caused a rise of about 10 mV in the *Eh* of milk from the 'evacuated' bottle and a decrease in the poising capacity of the milk (as shown by the slope of curve 2 in Fig. 7*a*, *b*). The effect of air was more marked with milk from the 'open' bottle which showed complete loss of reducing capacity. When the nitrogen was replaced by air the *Eh* from the milk in the 'open' bottle rose immediately but there was a slight delay before the potential rose in milk from the 'evacuated' bottle.

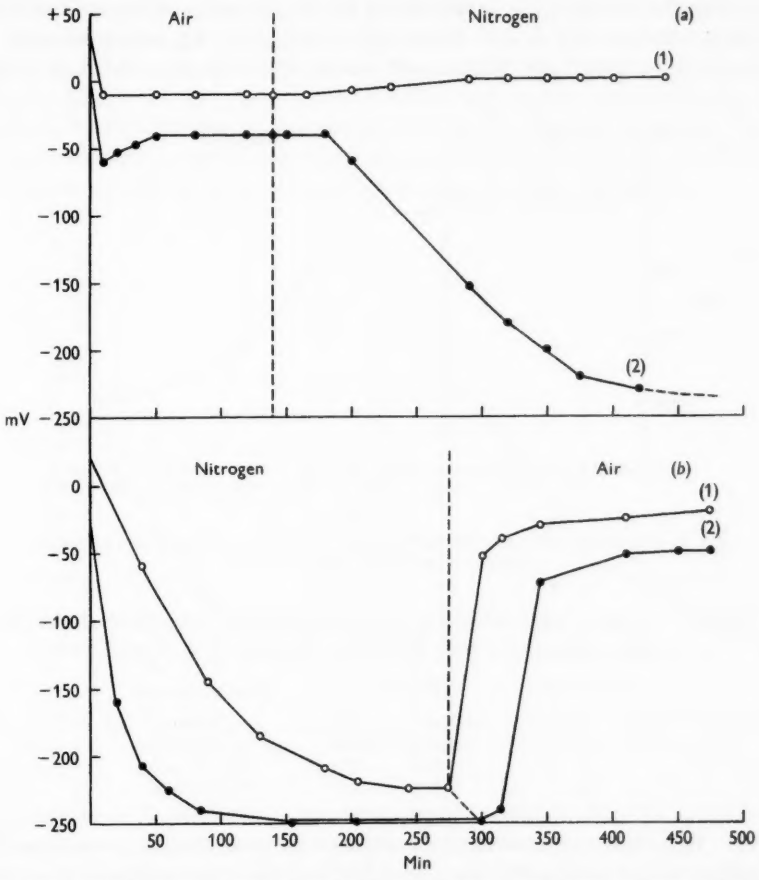

Fig. 7. Potential-time curves for milk sterilized by methods A and D and measured (*a*) in air and then under nitrogen, (*b*) under nitrogen and then in air. Curve 1, milk D; curve 2, milk A.

The results shown in Fig. 7 were typical of milks which did not readily cause a prolongation of the lag phase in the growth of mesophilic *Bacillus* species (Higginbottom & Taylor, 1960*a*). With milks which did show inhibition of growth there was a greater resistance to atmospheric oxidation. With such a milk there was a marked difference when measurements in milk heated in 'open' bottles were made first in air and then in nitrogen (Fig. 8). The resultant potential-time curve was very similar to that obtained with milk from an 'evacuated' bottle under the same conditions

(Fig. 7*a*, curve 2) but the limiting potential was about 100 mV more positive, about −150 mV as compared with −240 mV.

The potential of the milks became more positive on storage at 5 °C. This was associated with a measurable decrease in the oxygen-sensitive AFRS.

It is possible from these results to deduce something of the conditions present in the milk under the treatments A, B, C and D used in the bacteriological studies (Higginbottom & Taylor, 1960*a*). These treatments consisted of 'evacuated' bottles with commercial nitrogen (A) or air (B) in the headspace, and 'open' bottles with commercial nitrogen (C) or air (D) in the headspace. *Eh* measurements under nitrogen made on milk from 'evacuated' bottles represent the conditions occurring

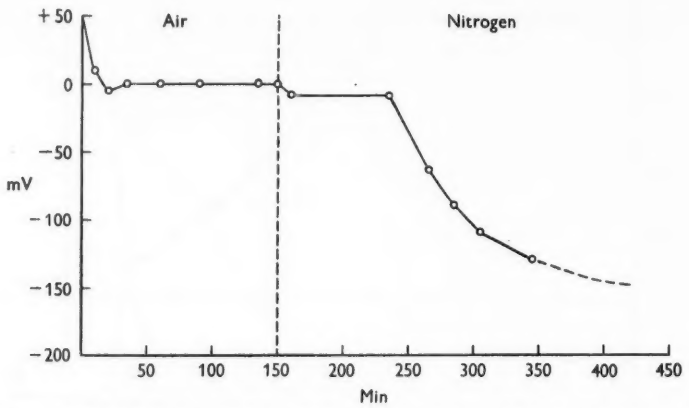

Fig. 8. Potential-time curve for milk sterilized in an 'open' bottle and measured first in air and then under nitrogen.

Table 1. *Approximate values for the oxidation-reduction potential and the poising capacity of milk treated by methods A, B, C and D*

Treatment	*Eh*, mV	Poising capacity
A	−280 to −300	Good
B	−260 to −280	Diminished
C	0 to −150	Poor or none
D	0 to < −150	Poor or none

in milk A. In milk B, the amount of air available to cause oxidation would be limited (the volume of the headspace was about 10% of the total volume), therefore the potential would be slightly more positive than in milk A but the poising capacity would be diminished, giving a response similar to curve 2 in Fig. 7*a*. The conditions prevailing in milk C will probably vary between a moderately low potential with little poising capacity (Fig. 8) and a relatively high potential with no reducing capacity (Fig. 7*a*, curve 1) depending on the milk. In all circumstances milk D will have a more positive potential and a poorer poising capacity than milk C. Approximate values for the different treatments are given in Table 1.

There was sufficient difference in *Eh* between treatments A and B and treatments C and D to account for different growth responses when small numbers of spores

were incubated in milk treated by these methods. The fact that *B. cereus* 201 and *B. brevis* 58 showed delayed growth in milks A and B but not in C and D supports this theory. Furthermore, variation in inhibitory powers of the milk as the degree of heat treatment increased could be demonstrated only to a slight degree with one strain, *B. brevis* 58. Of the non-biological properties studied only the potential and poising capacity of the milk remained constant over the range of heat treatments studied. As the size of the inoculum was increased there was a greater possibility that the spores might alter the micro-environment sufficiently to permit growth. Milk A had a better poising capacity than milk B and it was demonstrated with *B. subtilis* 6 and *B. cereus* 201 that growth may occur readily in milk B but be delayed in milk A. As the inoculum was increased still further growth occurred under all four conditions. While this cannot be regarded as proof that the inhibition was due to the oxidation-reduction potential *per se*, it is a strong indication that there was an association between inhibition of growth and oxidation-reduction compounds.

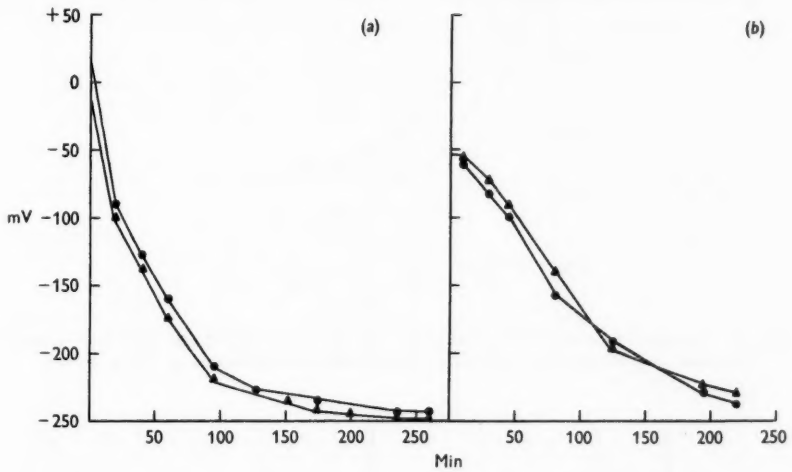

Fig. 9. Potential-time curves for duplicate samples of two commercial sterilized milks from plant A measured under nitrogen. (*a*) Milk processed 2, xii, (*b*) milk processed 9, xii.

The position of *B. circulans* is not incompatible with this theory. Leifson (1931) has shown that this species grows under more anaerobic conditions than other members of the genus, and presumably the optimal *Eh* for *B. circulans* is much lower than for other members of the genus. This species also appears to require different potentials for germination and for growth—a requirement not unknown for other organisms.

Commercial sterilized milk

Intact bottles of commercial sterilized milk were received from two sources within 2–5 days of processing. At A the batch process (AS) was used. At B in addition to milk sterilized by the continuous process (BS), samples were also obtained of milk heated by the ultra-high temperature short-time method (UHT) and of such milk given further treatment by the continuous process (UHT-S).

Good agreement was obtained with milks sterilized on the same day for the *Eh* measured at 50 °C under nitrogen. Typical results are shown in Table 2 for duplicate bottles and also the mean value for all samples for each type of milk. Differences in *Eh* and in the potential-time curves (Fig. 9) for samples taken at different times may indicate variations in the processing or in the composition of the milk or in both. Milks treated by the three methods used at plant B showed differences in the

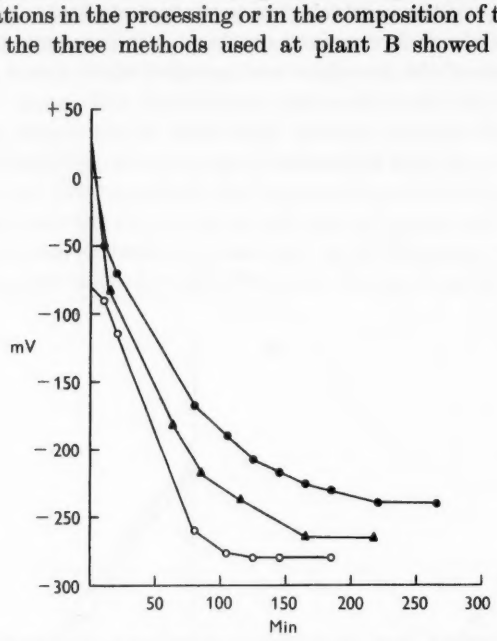

Fig. 10. Potential-time curves for UHT (○), BS (▲) and UHT-S (●) milk from plant B measured under nitrogen. UHT and UHT-S milk were processed on the same day.

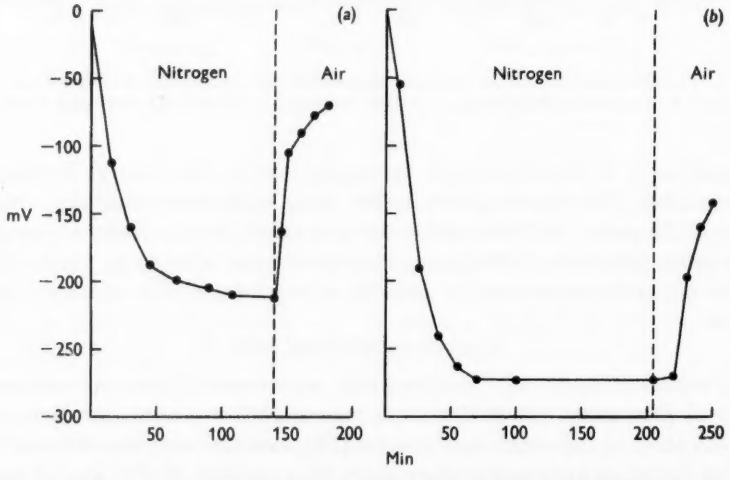

Fig. 11. Potential-time curves for batch sterilized milk from plant A (*a*), and UHT milk from plant B (*b*) measured first under nitrogen and then in air.

potential-time curves (Fig. 10) which suggest that the poising capacity of the milk decreased as the amount of processing of the milk increased.

The difference in poising capacity between UHT milk and milk sterilized by the batch process (AS) is shown in Fig. 11. The flow of nitrogen through the measuring cell was continued till a limiting *Eh* value was obtained and then it was replaced by air. The UHT milk showed a resistance to atmospheric oxidation similar to that obtained with milk heated in 'evacuated' bottles (Fig. 7), whereas there was an immediate rise in the potential of the batch-sterilized milk.

Table 2. *The* Eh *(at 50 °C under nitrogen) of commercial sterilized milk heated by the batch process (AS), the continuous process (BS), the ultra-high-temperature short-time method (UHT) and by UHT followed by in-bottle sterilization by BS (UHT-S)*

Process	Duplicate samples: Date	Duplicate samples: *Eh*, mV	Total number of samples	Mean *Eh* value for all samples, mV
AS	11. xi.	−240, −246	22	−238
	18. xi.	−237, −230	—	—
BS	18. xi.	−241, −229	12	−245
UHT	26. xi.	−280, −291	7	−273
UHT-S	30. ix.	−177, −171	7	−218

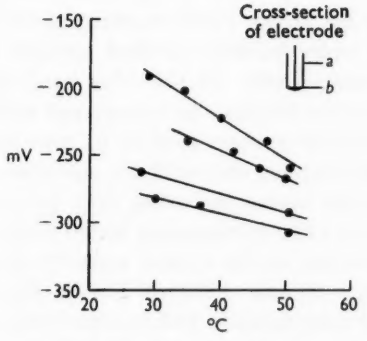

Fig. 12

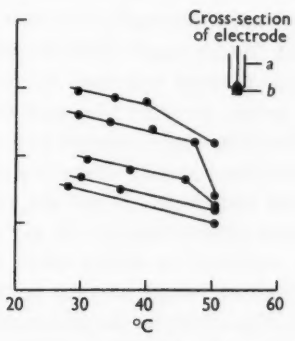

Fig. 13

Fig. 12. The relation of potential and temperature of measurement for platinum electrode no. 3. (*a*) glass tubing, (*b*) bright platinum.

Fig. 13. The relation of potential and temperature of measurement for platinum electrode no. 6. (*a*) glass tubing, (*b*) bright platinum.

The response of the electrodes at different temperatures

The values obtained for the *Eh* of sterilized milk were much lower than those previously reported for observations made at lower temperatures, and were also lower than those reported by Tobler (1955) using the same temperature (50 °C).

The type of electrode used may have influenced the results, and observations were therefore made on the effect of temperature and of different types of platinum electrode on the *Eh* of the sterilized milk determined under nitrogen. With the electrodes normally used consisting of a 5 mm disk of bright platinum fused to the end of a glass tube, a linear response was obtained over the range 30–50 °C (Fig. 12).

The gradient of the slope became greater as the potential of the milk became more positive within the range −260 to −310 mV. A second type of electrode consisting of a cylinder (2 × 3 mm diam.) of bright platinum fused into the end of a glass tube, gave a more complex temperature-potential relationship (Fig. 13). At temperatures between 30 and 45 °C there was a linear relationship with potential, but at temperatures above 45 °C there was a divergence from linearity which became more marked as the potential became more positive. The values for the *Eh* of sterilized milk, using the two types of Pt electrode, were identical at 50 °C but would not have been so had lower temperatures been used. Apart from the investigation of the temperature effect, the Pt electrodes used in this investigation were all of the same type.

When the response of the platinum electrodes to pH at 50 °C was investigated the family of lines obtained was very similar to that obtained with change in temperature (Fig. 12)—the rate of change was more rapid as the potential became more positive within the range −260 to −310 mV.

DISCUSSION

The oxidation-reduction potential of milk heated under partial vacuum and maintained out of contact with air, gave *Eh* values of the order of −200 to −300 mV. These values are very much lower than those given by Twigg (1937) for the *Eh* of autoclaved milk determined at 37 °C or by Tobler (1955) working at 50 °C. Both Twigg and Tobler made their readings under carefully purified nitrogen, whereas commercial nitrogen was used in the present work. On the other hand, particular care was taken, in order to create conditions referable to commercial sterilization, to heat the milk under vacuum and to exclude contact with air by maintaining the milk under (commercial) nitrogen during sampling and until the potential measurements were completed. When the milk was heated in bottles open to the air, the *Eh* in air was of the order of +30 mV falling when subsequently tested under nitrogen to about −150 mV or rather lower depending on the poising capacity of the milk. Tobler obtained values varying between −80 and −120 mV for the *Eh* of heated milk at 50 °C in nitrogen, the *Eh* becoming more negative with increase in the temperature of heating. Twigg reported initial values for *Eh* of +50 mV falling to −50 mV under nitrogen at 37 °C. The *Eh* values obtained by Twigg and by Tobler correspond most closely to those obtained with milk sterilized in open bottles when the potential was measured in nitrogen. Other factors such as the extent of the small potential leakage in the *Eh* meter or the type of electrode used may influence the value of the *Eh* recorded, but it seems clear that the low values obtained for milk sterilized under vacuum were due mainly to the care taken to exclude contact with air before the *Eh* measurements under nitrogen were determined. Greenbank & Wright (1951) reported lower *Eh* values for milk deaerated before heat treatment at 85–95 °C than in milk heated without deaeration. Harland, Coulter & Jenness (1952) also observed a marked reduction in the *Eh* of milk heated and tested in nitrogen compared with similar treatment in air using milk pasteurized at 85 °C for 30 min and *Eh* measurement at 30 °C.

We thank the Dairy Department of the Birmingham Co-operative Society Ltd. and the Edinburgh and Dumfriesshire Dairy Co. Ltd. for kindly supplying the samples of commercial sterilized milk.

REFERENCES

GREENBANK, G. R. & WRIGHT, P. A. (1951). *J. Dairy Sci.* **34**, 815.
HARLAND, H. A., COULTER, S. T. & JENNESS, R. (1952). *J. Dairy Sci.* **35**, 643.
HIGGINBOTTOM, C. & TAYLOR, M. M. (1960*a*). *J. Dairy Res.* **27**, 221.
HIGGINBOTTOM, C. & TAYLOR, M. M. (1960*b*). *J. Dairy Res.* **27**, 235.
LEIFSON, E. (1931). *J. Bact.* **21**, 331.
TOBLER, F. R. (1955). Eidgenössischen Technischen Hochschule, Zurich. Ph.D. thesis.
TWIGG, R. S. (1937). *J. Soc. Chem. Ind. Lond.*, **56**, 129.

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The alcohol test applied to sterilized milk

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Summary. A positive 80% alcohol test was not obtained in sterilized milks containing fewer than 10^5 bacteria/ml with *Bacillus subtilis*, *B. licheniformis* and *B. cereus*, and usually did not occur until the stationary phase of growth had been maintained for some days. A positive alcohol test with less than 10^5 bacteria/ml was associated with the lower maximum population density of *B. brevis* and *B. circulans* 152.

The alcohol test after 24 h at 37 °C was found unreliable as an indicator of the keeping quality of sterilized milk at 22 °C. The possibility of using the alcohol test after 3 days at 37 °C or a bacterial count after the same incubation period is discussed briefly.

There is no bacteriological standard for 'sterilized' milk in this country. Under commercial conditions here and in Holland, unopened bottles of milk are usually incubated for a definite time at 37 °C and then tested for bacterial growth by plate count. This method of testing is slow and under commercial conditions the batch of milk from which the sample bottles are taken will normally have been sold before the test is completed. Owing to the small numbers of surviving bacteria, some incubation of the milk is inevitable. The plate count introduces a further period of incubation. False results may be obtained due to the inhibitory effect of the plating medium on some strains of spore-forming rods. Inhibitory effects due to the medium have been shown for *Bacillus brevis* by Cowan (1953) and for some thermophiles by Taylor (1957).

To eliminate the time required to obtain a plate count, Morgan & Chalmers (1947) proposed a test using 80% alcohol following incubation of sterilized milk for 27 h at 37 °C as a means of assessing the potential keeping quality of the milk. The absence of a precipitate on mixing equal volumes of incubated milk and 80% alcohol was considered to indicate a keeping quality of at least 3 weeks when the milk was stored at atmospheric temperature. Morgan & Chalmers suggested that this test should be given further trials but no subsequent work on the subject has been published. The test has been used by Franklin, Williams & Clegg (1956) and by Hermier & Mocquot (1955) as an indicator of bacterial growth in sterilized milk, although it was appreciated that a negative test was not necessarily indicative of bacteriological sterility. Lack of stability to the addition of an equal volume of 80% alcohol was also used by Higginbottom & Taylor (1960) as an arbitrary end-point for observations on the growth of bacteria in sterilized milk. Although the

alcohol test was not studied primarily in relation to keeping quality, it is of interest to consider the relationship which was found between the results of the alcohol test and the growth of certain species of bacilli in sterilized milk.

EXPERIMENTAL

Full details of the treatment of the sterilized milk are given by Higginbottom & Taylor (1960). In brief, homogenized milk was sterilized in an autoclave at 115·5 °C for 15 min in bottles fitted with screw caps carrying a rubber diaphragm. Bottles of milk were either closed and evacuated before heating (milk A) or were heated open to the atmosphere and the caps screwed down after the milk was cool (milk D).

The sterilized milk was inoculated (through the rubber diaphragm) with very small numbers of spores of mesophilic spore-forming bacilli, incubated at the desired temperature and tested at intervals for bacterial content and by the alcohol test. For the alcohol test 1 ml of 80 % (v/v) ethyl alcohol, neutralized against phenolphthalein, was mixed with an equal volume of milk and the mixture immediately examined for precipitation.

RESULTS

The 80 % alcohol test was done each time a milk sample was taken for plate count in the studies on the growth of bacilli in sterilized milk (Higginbottom & Taylor, 1960). The numbers and results of the tests in relation to the log count/ml of the individual strains of bacilli for inoculated sterilized milk are shown as histograms in Fig. 1. Records of tests giving no precipitate with alcohol were more numerous than those which were positive since the milks were discarded shortly after the alcohol test became positive. These results take no account of the treatment of the milk, the size of the inoculum, or the time of incubation, but all refer to milk from fourteen farms which had been sterilized at 115·5 °C for 15 min and which, after inoculation, was incubated at 37 °C. With the exception of *B. brevis* 58 and *B. circulans* 152 positive alcohol tests were not obtained in milk containing fewer than 10^5 bacteria/ml. Considered in relation to the treatment of the milk (Fig. 2) these alcohol-positive low-count milks were usually associated with milk which was inhibitory to growth, i.e. for *B. brevis* 58 with milk which had been heated in 'evacuated' bottles (milk A) and for *B. circulans* 152 with milk which had been heated in 'open' bottles (milk D). With the other species of bacilli examined no appreciable difference between milks A and D was shown in the relationship of colony counts to the results of the 80 % alcohol test. As shown in Fig. 1 many milks with bacterial counts of 10^6 to 10^7/ml failed to show instability on the addition of 80 % alcohol. From an examination of the growth curves it was evident that the point at which the milk became unstable to alcohol usually occurred after the logarithmic phase, and for most strains studied the stationary phase was maintained for a period of days before the alcohol test was positive. Instability to alcohol thus depended not only on the numbers of bacteria present, but on the time for which these bacteria had been present in the milk. The relation between alcohol instability and the phase of bacterial growth is shown in Table 1. With *B. cereus* 201 the milk usually became unstable to alcohol just before the maximum population was attained and this was true also of two-thirds of the experiments using *B. circulans* 154. With

B. licheniformis 8 and *B. brevis* 58 a period of 2 or more days at 37°C after the maximum population was reached was required before the milk became unstable to alcohol. This accounts for the large numbers of negative alcohol tests recorded (Fig. 1) for these two strains of bacilli.

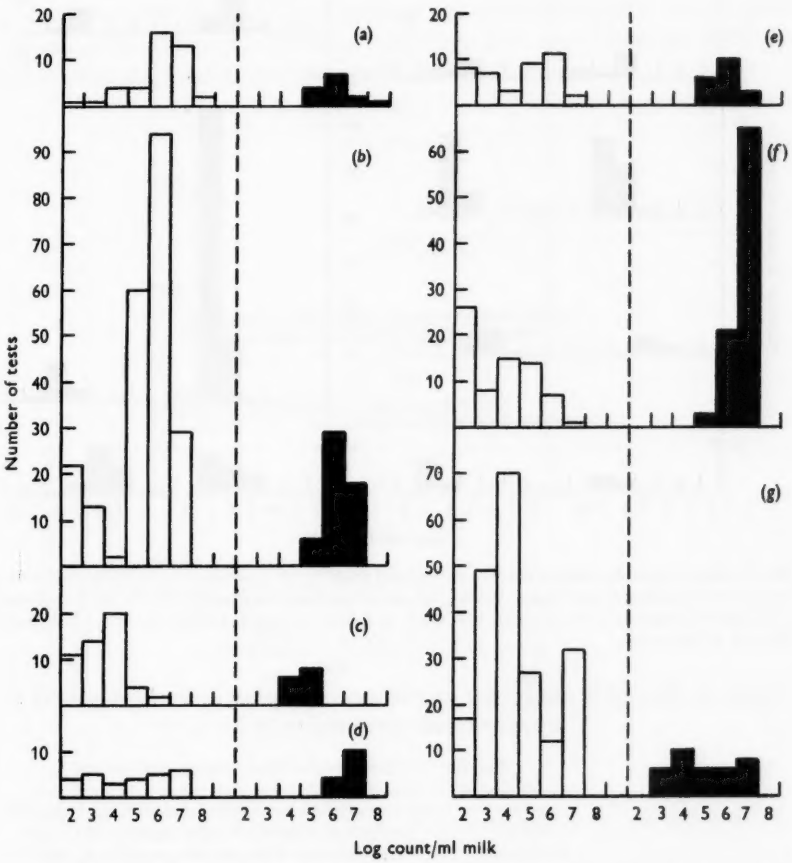

Fig. 1. The colony count/ml in relation to the 80% alcohol test in inoculated sterilized milk incubated at 37°C. (*a*) *B. subtilis* 6; (*b*) *B. licheniformis* 8; (*c*) *B. circulans* 152; (*d*) *B. circulans* 154; (*e*) *B. subtilis* D 1/2M; (*f*) *B. cereus* 201; (*g*) *B. brevis* 58. Test negative □, test positive ■.

With *B. brevis* 58 and *B. circulans* 152 the maximum population was lower in inhibitory sterilized milk (milk A for *B. brevis* and milk D for *B. circulans*) than in non-inhibitory milk (milks D and A, respectively). A longer period of incubation after the attainment of the maximum population was required in inhibitory than in non-inhibitory milk, to produce instability to alcohol. For instance, with *B. brevis* 58 the maximum population in the non-inhibitory milk D was 10^5 to 10^7 bacteria/ml. In inhibitory milk A the maximum population was 10^3 to 10^5 bacteria/ml and the milk required incubation at 37°C for 5–10 days longer than in milk D, to produce instability to 80% alcohol. The occurrence of positive alcohol tests in milks

containing less than 10^5 viable bacteria/ml was associated with inhibitory conditions in the milk which did not permit a maximum viable population of more than 10^5 bacteria/ml.

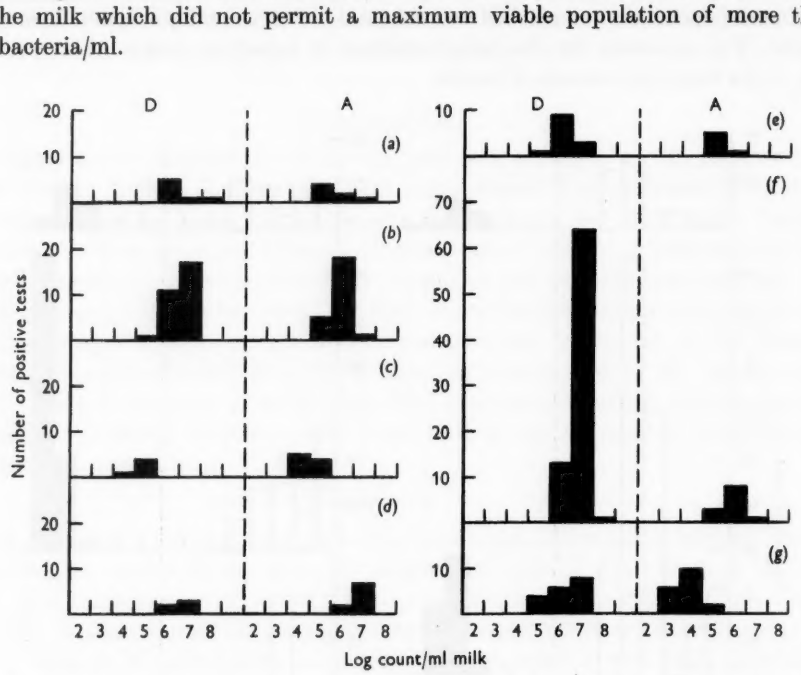

Fig. 2. The relation between colony count/ml and positive 80 % alcohol test in milk sterilized in 'evacuated' bottles (A) and 'open' bottles (D), inoculated and incubated at 37 °C. (*a*) *B. subtilis* 6; (*b*) *B. licheniformis* 8; (*c*) *B. circulans* 152; (*d*) *B. circulans* 154; (*e*) *B. subtilis* D 1/2 M; (*f*) *B. cereus* 201; (*g*) *B. brevis* 58.

Table 1. *The* 80 % *alcohol test in relation to the phase of bacterial growth in sterilized milk incubated at* 37 °C

	Number of experiments giving positive alcohol test						
	Before maximum population attained	After maximum population established. Period of incubation required to give positive test after establishment of maximum population, days					
Strain		<1	1	2	3–7	8–14	>14
B. subtilis 6	1	2	5	1	1	—	—
B. subtilis D 1/2 M	10	—	16	2	—	—	—
B. licheniformis 8	4	—	2	10	19	7	—
B. cereus 201	49	2	1	—	—	—	—
B. circulans 152	—	—	2	1	2	—	—
B. circulans 154	11	—	6	—	—	—	—
B. brevis 58	—	—	5	4	10	7	1

Morgan & Chalmers (1947) used an incubation period of 27 h at 37 °C before applying the alcohol test. Fig. 3 shows the time required to give a positive alcohol test in sterilized milk incubated at 37 °C after inoculation at various levels with each of the seven strains of bacilli used. The results are given for milks sterilized in both 'evacuated' and 'open' bottles, milks A and D. Under these conditions *B. cereus* 201

was the only strain which caused the milk to show instability on the addition of 80% alcohol within 27 h though this also applied in some instances to *B. subtilis* 6 in milk D, i.e. milk not inhibitory to the growth of this strain.

Using inocula varying from 4×10^{-3} to 600 spores/ml of milk the size of the inoculum within this range did not affect appreciably the incubation time at 37°C required to give a positive 80% alcohol test in non-inhibitory milks. As the inoculum was increased the difference between inhibitory and non-inhibitory milks in the incubation time required to produce alcohol instability decreased.

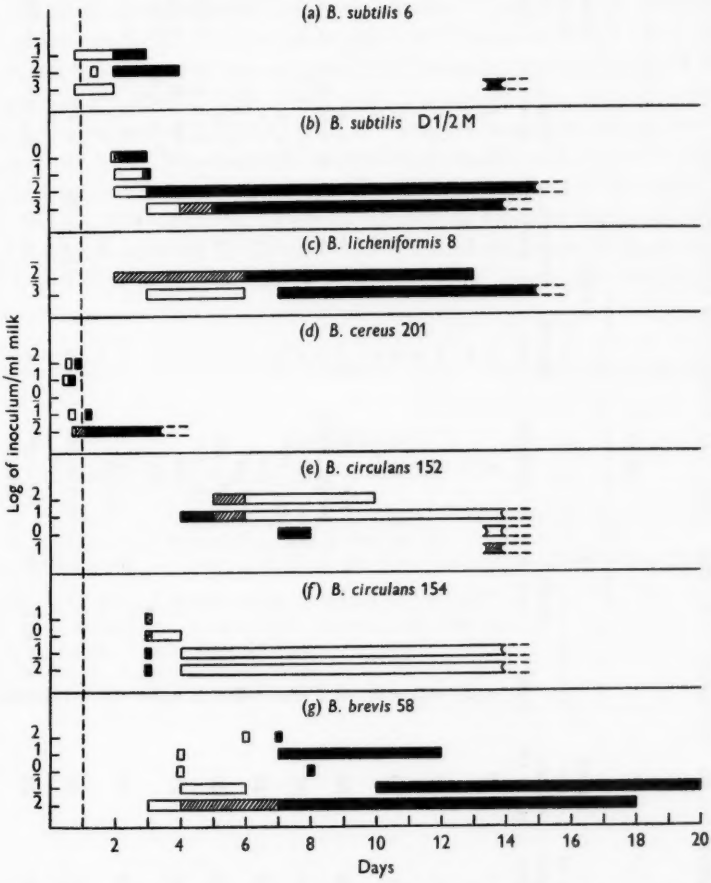

Fig. 3. Incubation time at 37°C required to give a positive 80% alcohol test in milk sterilized by methods A and D and inoculated with spores of bacilli. ■ milk A, □ milk D, ▣ overlap between milks A and D, □ stable to alcohol at end of observation period.

To obtain some indication of the value of the alcohol test at 37°C, as a measure of the keeping quality of sterilized milk, a comparison was made between bottles of the milk from the same batch given the same inoculum, incubated at 22°C and examined for growth and alcohol instability. The results are shown in Table 2. It is obvious

Table 2. *The relationship between the* 80 % *alcohol test and the plate count after* 1 *and* 3 *days at* 37 °C *and growth and alcohol test at* 22 °C *in milk sterilized in 'evacuated' (A) and 'open' (D) bottles and inoculated with very small numbers of bacillus spores*

Strain	No. of replicates	Spore inoculum/ml × 10^3	Milk	37 °C, 1 day: Alcohol test	37 °C, 1 day: Bacteria/ml	37 °C, 3 days: Alcohol test	37 °C, 3 days: Bacteria > 1000/ml	22 °C Growth: Onset, weeks	22 °C Growth: Presence or absence after 3 weeks	22 °C Growth: Maximum population, weeks	22 °C Growth: Alcohol test positive, weeks
B. subtilis 6	1	5	A	−	0	−	−	> 4	−	> 4	> 4
			D	−	10^7	+	+	1	+	1	1
B. subtilis D 1/2M (*a*)	1	20	A	−	0	−	−	> 4	−	> 4	> 4
			D	−	10	+	+	1	+	1	1
(*b*)	1	15	A	−	10	+	+	2	+	2	3
			D	−	10^2	+	+	1	+	1	1
B. licheniformis 8(*a*)	3	35	A	−	$10^3 - 10^4$	−	+	> 4	−	> 4	> 4
			D	−	10^3 (2), 0 (1)	−	+	> 4(2), 2(1)	−(2), +(1)	> 4 (2), 3 (1)	> 4(2), 4(1)
(*b*)	3	32	A	−	10^5	−	+	> 4, 2, 1	−(1), +(2)	> 4, 2, 1	> 4
			D	−	$10^3 - 10^5$	−	+	*> 4	−	> 4	> 4
(*c*)	3	36	A	−	10^2	−	+	> 4	−	> 4	> 4
			D	−	10^2	−	+	> 4(2), 2(1)	−(2), +(1)	> 4(2), 2(1)	> 4(2), 3(1)
(*d*)	3	28	A	−	$10^2 - 10^3$	−	+	> 4	−	> 4	> 4
			D	−	$10 - 10^3$	−	+	*> 4(1), 1(1)	−(1), +(1)	> 4(1), 1(1)	> 4(1), 3(1)
(*e*)	3	31	A	−	10^2	−	+	> 4(2), 2(1)	−(2), +(1)	> 4(2), 2(1)	> 4
			D	−	10	−	+	> 4, 2, 1	−(1), +(2)	> 4(1), 3(2)	> 4, 4, 3
B. brevis 58	2	18	A	−	0	−	−	4, 2	−(1), +(1)	8, 3	9, 5
			D	−	10^3	−	+	2, 1	+	3, 2	3, 3
B. cereus 201 (*a*)	3	40	A	−	0, 10^3, 10^5	+	+(1), −(2)	> 4	−	> 4	> 4
			D	+	10^6	+	+	1	+	1	1
(*b*)	3	16	A	+(1), −(2)	$10^3 - 10^6$	+	+	*2, > 4	+(1), −(1)	> 4, 2	> 4, 2
			D	+	10^6–10^7	+	+	*1	+	1	1

Where replicates differed numbers given in brackets.

* Two replicates only.

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from these results that a positive 80 % alcohol test after 24 h at 37 °C is of little value in predicting whether growth will occur at 22 °C within 3 weeks, except possibly with *B. cereus* which has been isolated only occasionally from commercial sterilized milk (Grinsted & Clegg, 1955; Ridgway, 1955; Candy & Nichols, 1956).

Tierie (1953) suggested that a satisfactory sample of sterilized milk should not clot on boiling, nor contain more than 1000 bacteria/ml after incubation at 37 °C for 3 days. The results obtained after 3 days at 37 °C are included in Table 2. For both strains of *B. subtilis* either a positive alcohol test or the presence of over 1000 bacteria/ml would serve as an indication of a poor keeping quality at 22 °C. With *B. licheniformis* 8 the alcohol test after 3 days was negative irrespective of whether or not growth occurred at 22 °C. On the other hand, the bacterial count after 3 days at 37 °C appeared to be too severe a standard for the assessment of growth at 22 °C. It appears that the strain of *B. licheniformis* used showed marked inhibition at 22 °C in milk A and did not always grow in milk D when the inoculum was of the order of 30–35 spores per litre. No observations have been made with larger inocula which might have been sufficient to overcome the inhibitory effect of the milk (Higginbottom & Taylor, 1960) at 22 °C. It seems unlikely, as has been shown in Fig. 3, that at 37 °C a moderate increase in the numbers of spores present will affect the alcohol test after 1 or 3 days or the number of milks containing more than 1000 bacteria/ml after 3 days.

We thank Mr P. B. McCluskey of Lochside Dairy, Ayr, for numerous samples of milk.

REFERENCES

Candy, M. R. & Nichols, A. A. (1956). *J. Dairy Res.* **23**, 329.
Cowan, S. T. (1953). *Lab. Pract.* **2**, 641.
Franklin, J. G., Williams, D. J. & Clegg, L. F. L. (1956). *J. appl. Bact.* **18**, 46.
Grinsted, E. & Clegg, L. F. L. (1955). *J. Dairy Res.* **23**, 178.
Hermier, J. & Mocquot, G. (1955). *Ann. Inst. nat. Rech. Agron. Paris Sér. E.* (*Ann. Tech. agric.*) **4**, 341.
Higginbottom, C. & Taylor, M. M. (1960). *J. Dairy Res.* **27**, 221.
Morgan, G. F. V. & Chalmers, C. H. (1947). *Nature, Lond.*, **159**, 510.
Ridgway, J. D. (1955). *J. appl. Bact.* **18**, 374.
Taylor, M. M. (1957). *J. gen. Microbiol.* **16**, ix.
Tierie, G. (1953). *Ned. melk-en Zuiveltijdschrift.* **7**, 138.

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Lactobacilli in raw milk and in Cheddar cheese

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Summary. The lactobacillus flora of the mixed morning and evening milk of a single herd and of sixty Cheddar cheese made from this milk, unheated, was examined over a period of 12 months. Few lactobacilli (usually not more than 1/ml) were found in the milk samples, and were identified as *Lactobacillus plantarum*, *L. casei*, *L. brevis*, *L. fermenti* and unclassified strains. In addition, *Pediococcus cerevisiae* and leuconostoc species were isolated on the selective medium used, the leuconostocs occurring more frequently than either the lactobacilli or the pediococci. The lactic-acid bacteria in the cheese varied considerably, both quantitatively and qualitatively. Although in general the same species of organisms were isolated from the cheese as from the milks, the relative frequencies of occurrence were not the same, leuconostocs being found much more often in milk than in cheese, and *L. plantarum* and *L. casei* more frequently in cheese. No association between numbers or types of lactic-acid bacteria and flavour of the cheese was noted, nor did the season of the year or the ration fed to the milking herd affect the lactobacillus flora of the milk.

The lactobacillus flora of Cheddar cheese is derived mainly from the milk. However, when the numbers in the milk are small the cheese flora may also be influenced by contaminants from the air and other sources (Naylor & Sharpe, 1958*a*). The work described here is concerned with the numbers and species of lactobacilli present in raw single herd milk and in cheese made from it. This work was done over a period of 12 months, to observe the effect of season, or changes in the diet of the milking herd, on the lactobacillus flora of the milk. Raw milk from a single herd was used as it was then in general use for cheesemaking at the N.I.R.D. experimental dairy, but a few cheese made from heat-treated milk from the same source were also examined, for comparison.

Isolations were made on a selective acetate agar medium. This medium, although valuable for isolating lactobacilli, is not completely selective, as it permits the growth of pediococci and some leuconostocs (Mabbitt & Zielinska, 1956; Leesment, 1956; Naylor & Sharpe, 1958*a*, *b*). However, the study of such organisms in milk and cheese is also of interest, as they may influence the cheese flavour if present in large numbers.

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METHODS

Cheesemaking. Mixed evening and morning milk from an Institute herd containing sixty to eighty milking cows was used for cheesemaking. Pairs of single-strain starters were used, but not in a particular rotation; the cheese were ripened at 55°F. Over the period October 1957–September 1958, inclusive, sixty cheese in all were set aside for examination. Each was 40 lb weight and made from 40 gal milk on different occasions. In the earlier part of the work, three cheese a week were examined, but later one cheese a week and finally only one or two each month were studied. Pairs of cheese made on the same day were occasionally included: with three pairs, raw and heat-treated (160 °F for 17 sec) milks were used, with three other pairs slightly different methods of manufacture were compared. The output from the dairy during the 12-month period averaged five 40 lb cheese per week.

Cultural conditions. Lactobacilli from milk and cheese were isolated and enumerated on the modified Rogosa acetate agar (AcA) of Mabbitt & Zielinska (1956). Plates were incubated anaerobically in an atmosphere of 90% H_2 + 10% CO_2. Incubation of all cultures was at 30°C.

Counts of lactobacilli in raw milk. The counts obtained by direct plating of the first few milks indicated that few lactobacilli were present, and several enrichment methods for enumerating the lactobacilli, based on those of Naylor & Sharpe (1958*a*), were compared (see Table 1). The milks, milks + starter streptococci and milks + acetate broth (AcB) were dispensed in screw-capped bottles, and incubated for 4 weeks. One per cent of starter was added, as in the cheese vat, to see whether the numbers of lactobacilli increased because of any stimulatory action of metabolites produced by the growth or lysis of the starter streptococci. Counts from direct plating on AcA were made after 5 days incubation. From enrichments, counts were made by streaking on to AcA, and incubating for 3 days. From the number of plates, and therefore of bottles, giving growth, the most probable number of lactobacilli in the original milks could be deduced by reference to McCrady's probability tables (Ministry of Health, 1939).

From the results of this preliminary work, it was decided that after 7 November 1957 counts and isolations should be made after plating 10 × 1 ml of milk on AcA and by adding 10 × 1 ml to 10 ml quantities of AcB and streaking on AcA after 5 days' incubation.

Counts of lactobacilli in cheese. Cheese were sampled after 6 weeks' ripening when the lactobacillus flora would have been approaching, or have already reached its maximum numbers (Mabbitt & Zielinska, 1956; Naylor & Sharpe, 1958*b*, *c*; Johns & Cole, 1959), and again after 15 weeks, when the maximum population would still have been present, or beginning to decline. Sampling and counting procedures used were those of Naylor & Sharpe (1958 *c*).

pH *of cheese.* To 12·5 g of minced cheese 17 ml water were added, and the pH determined electrometrically.

Flavour assessment of cheese. After 16 weeks' ripening, the flavour of each cheese was assessed by a tasting panel consisting of ten members of the Institute staff, some of whom were experienced in cheese tasting and others not.

Table 1. *Numbers and species of lactic-acid bacteria isolated from raw milk by direct plating and by enrichment methods*

	Direct plating of milk in Ac agar,	Milk alone,	Milk + starter from dairy,	Milk + starter from lab.,	Milk + acetate broth (AcB)		
	20 × 1 ml milk	1 × 50 ml 5 × 10 ml 5 × 1 ml	1 × 50 ml 5 × 10 ml 5 × 1 ml	1 × 50 ml 5 × 10 ml 5 × 1 ml	5 × (10 ml milk + 100 ml AcB)	1 × (50 ml milk + 50 ml AcB) 5 × (10 ml milk + 10 ml AcB) 5 × (1 ml milk + 1 ml AcB)	10 × (1 ml milk + 10 ml AcB)
Expt. no.	No./10 ml milk	MPN/10 ml milk	MPN/10 ml milk	MPN/10 ml milk	Proportion of bottles showing growth	MPN/10 ml milk	Proportion of bottles showing growth
1	2: B, Leu	1: B, Leu	—	—	5/5: B, U, Leu	—	—
2	1·5: Leu, B	0·7: Leu	—	—	2/5: Leu	—	—
3	3: Leu	0·5: (lost)*	—	—	2/5: Leu	—	—
4	3: Leu	No growth	0·8: P	0·7: Leu	—	0·5: U	2/10: Ped
5	0·5: (lost)*	No growth	No growth	No growth	—	No growth	1/10: Leu
6	27: Leu, Ped, B, P	1: B, P	0·8: Leu	0·5: B	—	1·7:Leu, P	9/10: Leu, Ped, P
7	2: Leu, F	0·3: Leu	1·1: F	0·4: Leu	—	0·6: Leu	1/10: Leu
8	No growth	0·7: (lost)*	0·3: Leu	1·4: F	—	0·8: Leu	1/10: Leu
9	No growth	0·3: Leu	0·5: Leu	0·7: Leu	—	0·3: B	1/10: Ped

MPN = most probable number; Leu = *Leuconostoc* spp.; Ped = *Pediococcus cerevisiae*; B = *Lactobacillus brevis*; P = *L. plantarum*; F = *L. fermenti*; U = unclassified streptobacteria which cannot be classified as either *L. casei* or *L. plantarum*. — =not tested.

* Failed to grow on further cultivation.

Table 2. *Differentiation of lactic-acid bacteria isolated from milk and Cheddar cheese on the selective acetate agar medium*

	Lactobacilli						
Microscopic appearance	*L. casei* Short G+rods in chains	*L. plantarum* G+rods	*L. fermenti* G+rods	*L. brevis* G+rods	*Leuconostoc* spp. G+cocci in chains, or coccobacilli	*Pediococcus cerevisiae* G+cocci in pairs, tetrads, clusters	*Micrococcus* spp. G+cocci in pairs, tetrads, clusters
Catalase	−	−	−	−	−	− or weak +	+
Growth at 15 °C	+	+	−	+	+	+	.
45 °C	−	−	+	−	−	−	.
Gas from glucose	−	−	+	+	+	−	.
NH_3 from arginine	−	−	+	+	−	±	.
YGLM*	ACR	ACR slow	A, AC	A, AC	AC s	AC slow	.
Growth in 0·1 % Teepol	−	+	−	±	±	+	.
Fermentation of:							
Mannitol	+	+	−	−	−	−†	.
Melezitose	±	+	−	−	−	−	.
Melibiose	−	+	±	−	−	−	.
Raffinose	−	+	±	−	−	−	.
Salicin	+	+	−	−	+	+	.
Trehalose	+	+	−	−	+	±	.
Xylose	−	+	−	+	±	+	.
Hydrolysis of aesculin	+	+	±	−	±	−	+
Growth on nutrient agar	.	.	.	.	.	−	+
Growth at pH 9·0	.	.	.	.	.	−	+
Final pH in glucose broth 4·5 or lower	.	.	.	.	.	+	−

* YGLM = yeast glucose litmus milk; s = often forms a stormy clot; . = not tested; ACR = acid, clot, reduction in 2 days; slow = taking more than 2 days to reduce.

† Of twelve representative strains which were examined, all fermented lactose, galactose and arabinose; only one strain fermented glycerol, and none fermented sucrose.

Isolation and identification of organisms from milk and cheese

(*a*) *Milk.* All the colonies growing from the milks plated directly on AcA and representatives of each type of colony appearing on plates inoculated from enrichment bottles (Table 1) were picked into AcB. After incubation the isolates were replated on tomato—glucose—agar, picked into tomato—glucose—broth and stored in yeast—glucose—litmus—milk.

(*b*) *Cheese.* Ten colonies were picked from AcA plates of suitable dilutions, purified and stored as above.

Strains were identified according to the scheme shown in Table 2, which is based on the criteria and methods used by Naylor & Sharpe (1958*a*). Since it soon became evident that pediococci and leuconostocs were occurring almost as frequently as lactobacilli, their differential characteristics are included. Pediococci are clearly distinguished from micrococci by their failure to grow at pH 9·0, their final pH in a glucose broth, and their inability to grow on a nutrient agar without adequate carbohydrate (Garvie, 1959).

Folinic acid requirements of pediococci. Twelve strains of pediococci isolated from different cheese were tested for their folinic acid requirements (Jensen & Seeley, 1954). Three serial transfers were made in the medium of Ford, Perry & Briggs (1958), with and without folinic acid. A requirement was denoted by absence of growth in the final transfer tube of the series lacking the growth factor.

RESULTS

The lactobacilli and lactic cocci isolated from all the milks and cheese are given in Table 3. The lactobacilli were identified as *Lactobacillus plantarum, L. casei, L. brevis* and *L. fermenti*. Several unclassified, low-temperature homofermentative strains were also present. These organisms differed from *L. casei* in microscopic appearance and in failing to ferment mannitol, melezitose, and usually trehalose. The pediococci were identified as *P. cerevisiae* (Pederson, 1949; Jensen & Seeley, 1954), but unlike the organisms described by the latter authors, not all strains formed NH_3 from arginine. They differed from the pediococcus isolated from cheese by Dacre (1958) in fermenting trehalose and in growing in the presence of 0·1 % Teepol. All twelve strains of pediococci tested were found to require folinic acid for growth. The leuconostocs were considered to be most closely related to *L. mesenteroides* (Garvie, unpublished data).

Milk. The numbers and species of lactic-acid bacteria isolated from milk by direct plating and by enrichment methods were similar (Table 1), confirming observations by Naylor & Sharpe (1958*a*), on milks with very low counts. It was considered that direct plating, together with a simple enrichment technique, would be all that was necessary for further examinations of milk.

Throughout this work the counts of lactobacilli and lactic cocci in the raw milks were low (Table 3); with ten of the fifty-three milks examined no lactobacilli were found in 20 ml. Only six milks had counts of 1/ml or greater, and in these most of the bacteria isolated were leuconostocs or pediococci. These organisms occurred in 65 and 30 %, respectively, of the thirty-seven milk samples from which isolates were

Table 3. *Numbers and species of lactobacilli and other lactic-acid bacteria isolated from raw milks and the Cheddar cheeses made from them, on a selective acetate medium*

Date cheese made	Milk		Cheese at 6 weeks		Cheese at 15 weeks	
	Count/ 20 ml	Organisms isolated	Count/g $\times 10^{-4}$	Organisms isolated	Count/g $\times 10^{-4}$	Organisms isolated
1957						
2. x.	0*	None	237	C, Ped	68	B
3. x.	0	None	12500	B, U	9800	C, P, B
9. x.	8	Leu	370	Ped, Leu	470	Ped
10. x.	2	Leu	97	P, Leu	1200	Ped
16. x.	2	Leu	138	B	89	P
22. x.	4	Leu, B, U	49	Ped, B	109	Ped, C
23. x.	3	Leu, B	55	Ped, B	4	Ped, B
24. x.	6	Leu	40	Ped, B	470	Ped, U
29. x.	6	Leu, P, U, Ped	22	Ped, U	26	Ped, P
30. x.	1	Leu	2	Ped, Leu	13	Ped, C
31. x.	54	Leu, Ped, B, P	13	Ped	23	Ped, B, U
5. xi.	4	Leu, F	3	Ped	0·003	U
6. xi.	2	Leu, F	< 1	None	2·2	Ped
7. xi.	3	Leu, B, Ped	9	Ped	0·055	P
12. xi.	16	Leu, Ped, U	3	P, U	24	Leu
13. xi.	11	Leu, Ped	14	P, U	57	Ped, Leu
14. xi.	20	P	28	Ped, P	195	Ped
19. xi.	6	B, P, U	64	P, U	165	P
20. xi.	2	P	0·002	Leu	0·155	Leu, B, P
21. xi.	50	Leu, Ped, B	7	Ped, P, U	6	Ped, Leu
26. xi.	15	Not identified	630	B, U, P	570	P, U, Leu
27. xi.	8	Not identified	460	B, P	530	B, U
28. xi.	8	Not identified	19000	C, P, U, B	2750	C, B, Leu
3. xii.	104	Leu, P	9900	P, B, Ped	570	P, Leu, Ped
4. xii.	9	Not identified	460	B, P	4650	C
5. xii.	11	Not identified	680	B, P	92	P, C, Leu
10. xii.	0	None	71	B	1330	B
11. xii.	0	None	3850	U	65	U
13. xii.	0	None	240	C, B	140	C
17. xii.	5	C, Leu	13	P	45	P
18. xii.	7	Leu, B, C, Ped	8	P	135	P, U
1958						
15. i.	3	Leu, P	0·15	Ped	111	P
22. i.	5	B, Ped	0·03	P, B	130	B, P
29. i.	24	Leu, B	1·06	B, Leu	760	B
6. ii. (*a*)	2	B, U	16·4	P, Ped	460	Ped
6. ii. (*b*)			5·5	Ped	820	Ped
13. ii. (*a*)	11	Leu, P, B	1·6	P, Ped	1280	B, Ped
13. ii. (*b*)			< 1	None	2340	B
20. ii. (*a*)	2	U	70	B, P, U	9800	C, Leu
20. ii. (*b*)			8·3	P, U	1450	P, B
27. ii. (*a*)	33	Leu, P, U, B	27	B, P, U	740	U, Leu
27. ii. (*b*)			0·2	Ped	430	U
6. iii. (i)	4	U	Unrecorded	P, B	140	B
6. iii. (ii)			Unrecorded	P, B	185	B
13. iii. (i)	3	P, U	2·4	Ped	700	P, C
13. iii. (ii)			<1	None	980	B
20. iii. (i)	3	F	96·5	C, B	280	P, C
20. iii. (ii)			1·4	P	81	P
17. iv.	3	B	0·14	Ped	< 1	None
23. iv.	3	Ped	2·85	B, Ped, Leu	52	U, C, Ped
8. v.	3	Ped	4·7	C, P, B	0·28	C, B
15. v.	0	None	17·6	P	84	B
22. v.	9	Not identified	0·03	B, U, P	3	Ped
12. vi.	0	None	35	Ped	128	B, Ped
2. vii.	0	None	92	B, U	7400	P, B
31. vii.	2	Leu	< 1	None	Unrecorded	B
2. ix.	0	None	4·30	P, Leu	11	P
4. ix.	7	Ped, B	2	Leu	2·5	P
9. ix.	0	None	46	B	12	P, B
10. ix.	7	B, Leu	20	B, P	227	B, P, Ped

	Milk	Cheese at 6 wks	Cheese at 15 wks
No. of samples examined	53	60	60
No of samples from which lactic-acid bacteria isolated and identified	37	56	59
Total no. of isolates	74	103	96

* 0 = No lactic-acid bacteria isolated from 20 ml of milk; B = *Lactobacillus brevis*; P = *L. plantarum*; C = *L. casei*; U = Streptobacterium with indeterminate characteristics; Ped = *Pediococcus cerevisiae*; Leu = *Leuconostoc* spp.; (*a*) = raw milk used for cheesemaking; (*b*) = same batch of milk, pasteurized at 160°F for 17 sec used for cheesemaking; (i) = raw milk cheese made in normal way; (ii) = another raw milk cheese made from the same batch of milk, using slightly different cheesemaking technique.

obtained (Table 4). *L. brevis* was the lactobacillus occurring most frequently (40 % of the samples), *L. plantarum* and unclassified lactobacilli occurred in about a quarter of the samples, and *L. casei* and *L. fermenti* were found only infrequently. No changes occurred in the lactobacillus flora of the milk when the rations fed to the milking herd were changed from kale to silage, when hay was given, or when the herd was put out to grass in the spring. Seasonal changes, also, had no effect on the flora, although as the summer of 1958 was cool, temperature effects might have been less than in a normal season.

Cheese. In cheese the viable counts on AcA varied greatly, from 20/g to $1{\cdot}9 \times 10^8$/g in the young cheese, and from 30/g to $9{\cdot}8 \times 10^7$/g in the older cheese (Table 3). Counts increased between the 6 and 15 weeks samplings in more than half the cheese, but remained relatively constant, or decreased in others. There was no correlation of the higher count milks with higher counts in the cheese. Variations in the counts from day to day were of the same order as those from summer to winter. There was no association between numbers of organisms present and those of any particular species of lactobacillus or lactic coccus. *L. plantarum*, *L. brevis* and pediococci were the organisms isolated most frequently (Table 4) from both 6 and 15 weeks old cheese.

Table 4. *Distribution of species of lactic acid bacteria in samples of milk and cheese (excluding samples containing no lactic acid bacteria)*

	Milk		Cheese			
			6 weeks old		15 weeks old	
Organisms isolated	No.	%	No.	%	No.	%
Leuconostoc spp.	24	65	8	14	10	17
P. cerevisiae	11	30	22	39	20	34
L. plantarum	10	27	28	50	22	37
L. brevis	15	41	26	46	22	37
Unclassified	9	24	14	25	10	17
L. casei	2	5	5	9	12	20
L. fermenti	3	8	0	0	0	0
Total number of samples containing lactic-acid bacteria	37		56		59	

L. casei was found only occasionally in the young cheese, but more often in the older ones. Leuconostocs were isolated much less frequently than from milk. As expected, no strains of *L. fermenti* occurred, the ripening temperature of the cheese being below the minimum growth temperature of this organism. Only seldom was the same flora found in the milk as in the cheese, although usually some of the species were the same; rarely did a cheese have the same flora at the two samplings. When two cheese were made from the same milk (raw and pasteurized), or when two raw milk cheese were made together, the flora was on some occasions the same or similar in the pairs, but on others quite different.

The range of pH in the cheese at 4 months was 4·8–5·2, so that some of the cheese were very acid. There was no agreement between numbers of organisms present in the 15 weeks old cheese and a high or low pH, nor between any of these factors and the flavour assessment of the cheese. The flavour of the cheese varied little during

the year, and proved to be disappointing. The general opinion, consistently expressed by the panel, was that most of the cheese were acid and bitter, and lacked true Cheddar flavour.

DISCUSSION

The lactobacilli isolated from milk and cheese on the selective acetate medium were identified by physiological tests only. As identification at species level was considered sufficient, serological typing was not used. Several strains, which could not be identified by physiological tests, were isolated: they appeared to be more closely related to *L. casei* than to *L. plantarum*. Many such indeterminate strains which cannot be classified as either *L. casei* or *L. plantarum*, because they have sufficient differentiating characteristics to separate them from both species, have been previously isolated from cheese (Sherwood, 1939; Naylor & Sharpe, 1958*a*, *b*).

The small numbers of lactobacilli found in the milks confirmed the findings of Jensen & Edmondson (1957) who found that only 75 of 225 samples of raw milk examined by direct and enrichment methods contained lactobacilli. Johns & Cole (1959) record slightly larger numbers, of more than 10/ml lactobacilli in two raw milks from their experimental farm. Hill & Thornton (1958) found no lactobacilli in two out of eleven samples of market milk they examined, but larger numbers, ranging from 10 to 10000/ml in the others. Such low numbers are in agreement with those recorded by Naylor & Sharpe (1958*a*) in the earlier part of their work. No increase in numbers, such as these latter workers found in the milk during November and December 1956, was noted in 1957, even though the milk was obtained from the same herd and under similar conditions of management; this increase in numbers in 1956 may have been due to some chance contamination with larger numbers of lactobacilli.

The lactobacilli isolated from the milks were, with the exception of *L. fermenti*, all low-temperature organisms, capable of proliferating in milk and cheese. The infrequent occurrence of *L. casei* in the milk, compared with that of the other species isolated, is unexpected. Jensen & Edmondson (1957) found *L. casei* in 57 % of their raw milks which contained lactobacilli, whilst *L. plantarum* and *L. brevis* occurred in only 15 and 0·26 %, respectively. Hill & Thornton (1958) also found the lactobacillus flora of the milks they examined to consist mainly of *L. casei*, but did not differentiate this organism from *L. plantarum*. However, these observations are somewhat invalidated, as both these groups of workers included in the genus *Lactobacillus* only organisms which, among other characteristics, reduced and coagulated litmus milk or glucose—yeast extract—chalk—litmus—milk. This criterion excludes many strains of *L. brevis* from the genus, as this species may show no reaction or only acid in these media, even after 14 days' incubation at 30 °C.

The lactic-acid flora of the milks examined here consisted mainly of leuconostocs, pediococci, *L. brevis* and *L. plantarum*. In a commercial dairy different conditions might lead to a different residential flora containing greater numbers of *L. casei*. Perry, Sharpe & Mattick (1958) found that this species was by far the most common lactobacillus in the air of commercial creameries, whereas sampling of air in the Institute dairy during cheesemaking on different occasions resulted in the isolation of *L. plantarum*, *L. brevis*, unclassified homofermentative lactobacilli (similar to those

described here from the milk), leuconostocs and pediococci. Naylor & Sharpe (1958*a*) have previously shown that serological types of lactobacilli found in the air may also occur in the cheese milk and cheese.

The great variation in lactobacillus counts in some of the cheese examined may be explained by irregularity of dispersion of the bacteria in the cheese. Hiscox, Harrison & Wolf (1940) found that the bacteria in Stilton cheese were concentrated in irregularly scattered colonies, and Dean, Berridge & Mabbitt (1959) have shown by means of photographs of stained sections that the same is true of Cheddar cheese. The very small numbers of lactobacilli and lactic cocci encountered in the milk during our investigation must also have led to irregular distribution in the cheese and may account for the difference in our findings from those of Johns & Cole (1959) and Feagan & Dawson (1959), who did not observe such fluctuations in numbers.

Although the lactic-acid bacteria isolated from the cheese were, with the exception of *L. fermenti*, the same species as those found in the milk, the proportionate numbers of the different species were not the same. Leuconostocs were isolated four times as frequently from milk as from cheese, whilst *L. plantarum* and *L. casei* were isolated about twice as often from cheese as from milk. This suggests that lactobacilli increase much more rapidly than leuconostocs in the cheese, and *L. casei* and *L. plantarum* more rapidly than *L. brevis*.

It was not possible to show any influence of particular species of lactobacilli on flavour, in spite of variations in numbers and species, as the flavour was consistently somewhat unsatisfactory.

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REFERENCES

Dacre, J. C. (1958). *J. Dairy Res.* **25**, 409.
Dean, M. R., Berridge, N. J. & Mabbitt, L. A. (1959). *J. Dairy Res.* **26**, 72.
Feagan, J. T. & Dawson, D. J. (1959). *Aust. J. Dairy Tech.* **14**, 59.
Ford, J. E., Perry, K. D. & Briggs, C. A. E. (1958). *J. gen. Microbiol.* **18**, 273.
Garvie, E. I. (1959). *Nature, Lond.*, **183**, 1411.
Hill, D. A. & Thornton, H. R. (1958). *Canad. J. Microbiol.* **4**, 215.
Hiscox, E. R., Harrison, J. & Wolf, J. Z. (1940). *Proc. Soc. agric. Bact.* p. 23.
Jensen, E. M. & Seeley, H. W. (1954). *J. Bact.* **67**, 484.
Jensen, R. G. & Edmondson, J. E. (1957). *J. Dairy Sci.* **40**, 180.
Johns, C. K. & Cole, S. E. (1959). *J. Dairy Res.* **26**, 157.
Leesment, H. (1956). Inaug. Diss., Kiel.
Ministry of Health (1939). The Bacteriological Examination of Water Supplies. *Rep. publ. Hlth. med. Subj. Lond.*, no. 71. London: H.M.S.O.
Mabbitt, L. A. & Zielinska, M. (1956). *J. appl. Bact.* **19**, 95.
Naylor, J. & Sharpe, M. E. (1958*a*). *J. Dairy Res.* **25**, 431.
Naylor, J. & Sharpe, M. E. (1958*b*). *J. Dairy Res.* **25**, 92.
Naylor, J. & Sharpe, M. E. (1958*c*). *J. Dairy Res.* **25**, 421.
Pederson, C. S. (1949). *Bact. Rev.* **13**, 225.
Perry, K. D., Sharpe, M. E. & Mattick, A. T. R. (1958). *J. Dairy Res.* **25**, 407.
Sherwood, I. R. (1939). *J. Dairy Res.* **10**, 426.

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Lactobacilli in milk used for commercial cheesemaking

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Summary. The numbers of lactobacilli, including pediococci and some leuconostocs, in raw and heat treated milks at twenty-six cheese factories in different parts of the country were determined over a period of 12 months, by plating on acetate agar. Counts in the raw milks varied from < 1 to > 100000/ml, but were usually within the range 100 to 10000/ml. Widely different times and temperatures of heat treatment were in use at the different creameries, some of which destroyed most of the lactobacilli present, whilst others had less effect. Post heat treatment re-infection of the milk in the vat with lactobacilli usually occurred.

The old problem of the exact role of lactobacilli in cheese ripening and in flavour production persists. But the work of Naylor & Sharpe (1958) and Johns & Cole (1959) suggests that the lactobacillus flora of the milk in the cheese vat is likely to determine that of the cheese made from it. The work reported here was primarily intended to be an enumeration of lactobacilli in factory cheese milk, using the selective acetate agar of Mabbit & Zielinska (1956) for the purpose. This medium, however, also allows the growth of *Pediococcus cerevisiae* and of some leuconostocs, which may occur in raw milk and in cheese in numbers equal to those of the lactobacilli (Perry & Sharpe, 1960). That these organisms grow on an acetate medium suggests a metabolism similar to that of the lactobacilli and it may well be useful to know the numbers of lactobacilli, leuconostocs and pediococci in milk used for cheesemaking. Dacre (1958) found that pediococci were the dominant flora of some New Zealand Cheddar cheese, in which he thought they might contribute to the flavour. The term 'lactobacillus' throughout this paper refers to the total numbers of colonies developing on acetate agar. The count may include leuconostocs and pediococci, as well as lactobacilli.

Lactobacilli were enumerated in raw milks, the same milks directly after heat treatment, and again in the vat just before cheesemaking started. The data obtained showed the numbers present in bulked raw milk over a wide area of Great Britain, the seasonal variation in numbers in the raw milk, the effect of the various heat treatments used at different creameries, the degree of re-infection with lactobacilli which might occur, and the numbers of lactobacilli present in the milks at the beginning of cheesemaking.

The work was done in collaboration with the National Agricultural Advisory Service, and the West of Scotland Agricultural College, who were responsible for the

milk sampling and plate counts and for recording the information from the different creameries. Five N.A.A.S. regions, where creamery cheesemaking takes place, took part in this work, namely, Wales, South West, East Midland, West Midland, and Northern.

METHODS

Twenty-six creameries participated; at seventeen of them samples were taken over a period of 12–13 months, whilst at the other nine only part of the year was covered. The investigation extended from August 1957, to September 1958, inclusive. Milk samples were usually taken at monthly intervals or occasionally at weekly or fortnightly intervals.

Samples of the raw milk, the heat-treated milk directly after leaving the pasteurizer, and of the same milk from the vat, were examined within a few hours of sampling, at the nearest National Agricultural Advisory Service laboratory. Duplicate quantities of 1·0 ml and dilutions of 1/10 and 1/100 were plated on the modified Rogosa acetate agar of Mabbitt & Zielinska (1956). Plates were incubated anaerobically in an atmosphere of CO_2 for 5 days at 30 °C before counting. Acetate agar prepared at this Institute was used throughout.

The temperature of heat treatment at the different creameries was read from the thermometer or the recording thermometer attached to the pasteurizer at each sampling. Plate pasteurizers were in use at all the creameries.

RESULTS

The numbers of samples and intervals of sampling were sometimes irregular, and the times and temperatures of heat treatment were variable, so that only general information about the creameries can be given (Table 1).

Counts of lactobacilli in raw milks. The numbers of samples taken at each creamery are shown in Table 1, and the distribution of the lactobacillus counts in Fig. 1. The counts of all the raw milks have been presented together. About 18 % of the samples had counts of less than 100/ml, 76 % had counts between 100 and 10000/ml, and the remaining 6 % contained 10000 to 1000000 lactobacilli/ml. These figures are in the same range as those of Johns & Cole (1959), who found a mean of 990 lactobacilli/ml in ten Canadian market milks, but differ from those of Jensen & Edmondson (1957) and Hill & Thornton (1958), who recorded very small numbers of lactobacilli in the market milks they examined.

In none of the seventeen creameries where the sampling had extended over the whole 12-month period was any clear-cut seasonal trend in the lactobacillus counts evident, although there was perhaps a general tendency for greater numbers of lactobacilli to occur in the milks during the summer months. The highest counts in the greatest number of milks were in August, May and September, whilst the lowest counts were in general in January and November. The summer of 1958 was cool and wet, so that differences in seasonal temperature were less than might occur in more normal seasons.

Counts of lactobacilli in heat-treated milks. The heat treatment varied from one creamery to another (Table 1), some flash heating at 150 °F, others heating the milk

Table 1. *Heat treatment of the cheese milks sampled at creameries in different parts of Great Britain*

Creamery	Situation of creamery	Types of cheese mainly produced	Gal milk/day used for cheese-making $\times 10^{-3}$ *	No. of samples of raw milk examined	Heat treatment of milk: Time, sec	Heat treatment of milk: Temperature, °F: Range	Heat treatment of milk: Temperature, °F: Mean
A	Wales	Cheshire	13	16	15	155–163	162
B	Wales	Cheshire	40	22	Flash	153–170	164
C	Wales	Cheshire	10–15	9	15	145–155	152
D	Wales	Cheddar, Caerphilly	20–25	10	Flash	156–164	161
E	Wales	Cheshire	5–7	13	15	162	162
F	Wales	Caerphilly	15–20	10	Flash	162–171	164
G	Wales	Cheddar, Cheshire	20–30	18	(*a*) Flash	162–164	164
					(*b*) 15	160–161	160
H	Wales	Cheddar	20–30	13	15	146–165	159
Z†	Wales	Caerphilly, Cheddar, Cheshire	variable	6		Variable	
J	S.W. England	Cheddar, Caerphilly	14	8	15	162–164	163
K	S.W. England	Cheddar	6	9	15	161–164	163
L	S.W. England	Cheddar	12	8	10–15	161–162	161
M	S.W. England	Cheddar	3	9	15	151–155	152
N	S.W. England	Cheddar	2	10	15	162	—
O	S.W. England	Cheddar	59	10	10–15	150–165	154
R†	E. Midland	Cheshire, Cheddar	5	8	12–15	162	—
S†	E. Midland	Cheddar	3	6	15	150–155	154
P	W. Midland	Cheshire, Cheddar	21	9	15	145–155	149
Q	W. Midland	Cheddar	25	8	10–15	145–155	151
T†	W. Midland	(Farm House) Cheshire	0·25	5		Raw milk used	
U†	W. Midland	Cheshire	4	7		Raw milk used	
V†	W. Midland	Cheshire	15	0‡	Flash	168–170	168
W†	W. Midland	Cheshire	40	7	Flash	158–160	159
I	N. England	Cheshire	14	25	15	155–159	156
Y†	N. England	Cheddar, Cheshire	20–30	20	15	150	—
X†	W. Scotland	Cheddar, Dunlop	13	7	Flash	150	—
			Total	273			

† Milks sampled over only part of the 12 month period of investigation.
* At peak milk production period.
‡ Only heat treated milk in the vat sampled.

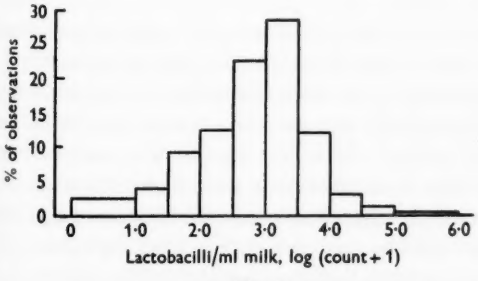

Fig. 1

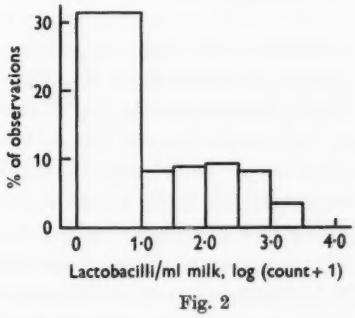

Fig. 2

Fig. 1. Distribution of numbers of lactobacilli in 273 samples of raw milk.

Fig. 2. Distribution of numbers of lactobacilli in 260 of the same samples of milk as Fig. 1, directly after heat treatment.

for 15 sec at over 160 °F. The temperature of heat treatment at a creamery also varied from one time of sampling to another, so the range and mean of the temperatures are included. The distribution of counts of all the heat-treated milks (Fig. 2), shows that the heat treatments used generally destroyed most of the lactobacilli, 65 % of the samples having counts of less than 10/ml. Fig. 3 gives the effect of heat treatment at each creamery separately. In many instances less than 1 lactobacillus/ml survived in the milk. In others the results were more variable. Sometimes lactobacilli were found in the milk after heating at temperatures higher than 161 °F for 16 sec; at one

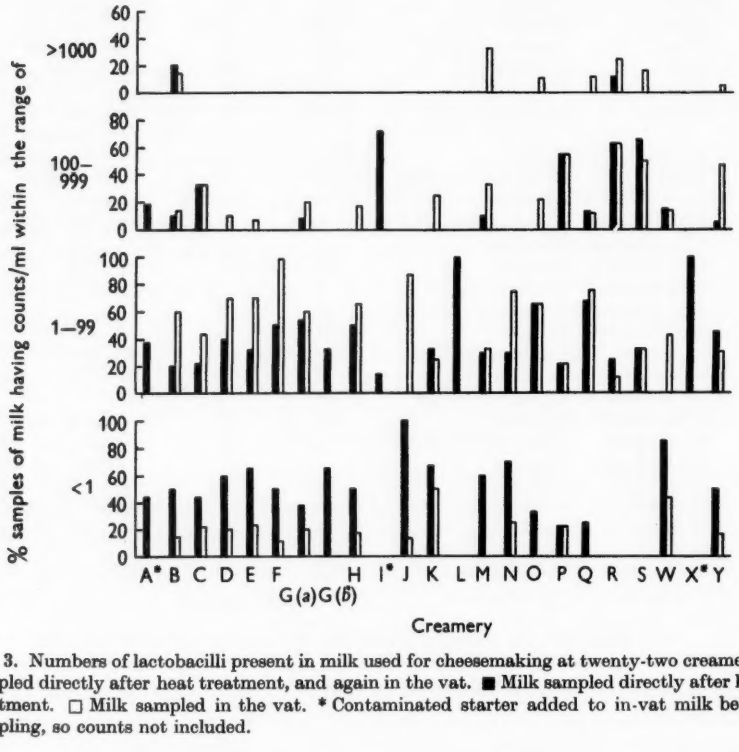

Fig. 3. Numbers of lactobacilli present in milk used for cheesemaking at twenty-two creameries sampled directly after heat treatment, and again in the vat. ■ Milk sampled directly after heat treatment. □ Milk sampled in the vat. * Contaminated starter added to in-vat milk before sampling, so counts not included.

creamery more than 1 % of the original number of lactobacilli were found after heating on one occasion, whereas on another, under the same conditions none survived. Flash heating even at 165 °F seldom destroyed all the lactobacilli. Cheese factories in the South West (J–O) and in Wales (C–H) showed the greatest reduction in numbers of lactobacilli in the heated milks. At a few creameries, particularly creameries R, S, P, V and I, counts after heat treatment were higher than usual. This was investigated further at creamery I, where the post-heat treatment counts were consistently high; after checking that the pasteurizer was working correctly, swabs were taken at the blind end of the pipeline leading from the pasteurizer to the cheese vats. Counts of lactobacilli were approximately $4 \times 10^6/ft^2$. The milk after heat treatment was thus re-inoculated with lactobacilli from a reservoir which might equally well harbour undesirable micro-organisms or phage. The high counts in heat

treated milks at some of the other creameries may have been due to similar causes, and the term 'survivors' as used here must be interpreted with caution.

Lactobacillus counts of milk in the vat. Milk was resampled in the cheese vat just before or just after the starter had been added. The counts of lactobacilli were higher than they were immediately after the milk was heat treated (Fig. 3), because of recontamination from the air (Naylor & Sharpe, 1958; Perry, Sharpe & Mattick, 1958), from dairy utensils (Sherwood, 1940) or from contaminated pipelines, and possibly because of the growth of lactobacilli surviving in the cheesemilk. In some of the creameries where the milk was sampled after the starter had been added, large increases in the lactobacillus counts of the milk often occurred. Sharpe *et al.* (1958) have since shown that these greatly increased numbers of lactobacilli came from the starters, which were heavily contaminated with lactobacilli. In-vat milk counts of samples taken after the addition of starter which was later shown to be contaminated, have not been included in Fig. 3.

Plates from raw and heat-treated milk samples which showed the presence of large and small colonies were sent to our laboratory on several occasions. Representative small colonies were picked and identified as *L. mesenteroides*, which was no doubt frequently present in the milks.

At all the creameries the quality of the cheeses produced was satisfactory, being graded as 'selected' or 'highly selected'. There appeared to be no association between quality of the cheese at the time of grading and high or low counts of lactobacilli in the cheese milk at a particular creamery.

DISCUSSION

The numbers of lactobacilli surviving heat treatment at the longer times and higher temperatures were often greater than those obtained by Perry & Sharpe (1959) who found that heat treating milk at 160 °F for 17 sec destroyed all the lactobacilli of a heavy inoculum added to the milk. Johns & Cole (1959) also found that on pasteurizing factory milk at 161 °F for 16 sec all the lactobacilli were destroyed. However, both these reports described work done in experimental dairies where conditions may be more rigidly controlled.

It was shown at one dairy that recontamination of the milk with lactobacilli occurred after the heating section of the pasteurizer, but before the sampling point. There is, therefore, no conclusive evidence from this work of the existence of strains more heat resistant than those studied previously under experimental conditions.

Although the numbers of lactobacilli were greatly reduced by the heat treatment, the number of samples in which milk in the vat at the start of cheesemaking contained less than 1 lactobacillus/ml was low. This was partly due to contamination in the vat, as shown by the counts of heated milk and milk taken from the vat. At six creameries no in-vat milks contained less than 1/ml. At the other thirteen some 22 % did. Only small volumes of milk were examined at each sampling, so that even if no lactobacilli were found on plating after heat treatment, this did not necessarily mean that all had been destroyed, but only that none was found in the 2 ml of samples examined. In our experience (Perry & Sharpe, 1960) milks with very low lactobacillus counts do not necessarily result in cheese with low lactobacillus counts. An average of 51 % of the total samples of in-vat milks contained 1–99 lactobacilli/ml.

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REFERENCES

DACRE, J. C. (1958). *J. Dairy Res.* **25**, 414.
HILL, D. A. & THORNTON, H. R. (1958). *Canad. J. Microbiol.* **4**, 215.
JENSEN, R. G. & EDMONDSON, J. E. (1957). *J. Dairy Sci.* **40**, 180.
JOHNS, C. K. & COLE, S. E. (1959). *J. Dairy Res.* **26**, 157.
MABBITT, L. A. & ZIELINSKA, M. (1956). *J. appl. Bact.* **19**, 95.
NAYLOR, J. & SHARPE, M. E. (1958). *J. Dairy Res.* **25**, 431.
PERRY, K. D., SHARPE, M. E. & MATTICK, A. T. R. (1958). *J. Dairy Res.* **25**, 407.
PERRY, K. D. & SHARPE, M. E. (1959). *J. Dairy Res.* **26**, 72.
PERRY, K. D. & SHARPE, M. E. (1960). *J. Dairy Res.* **27**, 267.
SHARPE, M. E., PERRY, K. D., MATTICK, A. T. R., ADAMS, R. P., DRUCE, R. G., EGDELL, J. W., THOMAS, S. B. & CRAWFORD, R. J. M. (1958). *J. Dairy Res.* **25**, 418.
SHERWOOD, I. R. (1940). *Rep. D.S.I.R., N.Z.* no. 135, p. 2.

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The genus *Leuconostoc* and its nomenclature

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SUMMARY. A preliminary examination of strains of leuconostocs collected from many different laboratories has resulted in a division into six groups. Some of these groups are heterogeneous. The name *Leuconostoc cremoris* is used in preference to *Leuconostoc citrovorum* for the non-sucrose fermenting species. Reasons are given. Other arguments lead to the conclusion that *Streptococcus kefir* (Evans) should be regarded as a synonym of *Leuconostoc mesenteroides*, and the name *Leuconostoc lactis* is used in preference to *Streptococcus kefir* (Abd-el-Malek & Gibson).

The species of the genus *Leuconostoc* have been known for many years as spoilage organisms in the sugar industry and as useful components of dairy starters. Reliable representatives of the different species for comparison with unknown strains are not easy to obtain, and it will be shown that in the past some cultures have been wrongly classified as leuconostocs. The American Type Culture Collection has made a number of alterations in the leuconostoc strains listed in the 6th edition of the catalogue (1958). Most of the strains are recent additions of *Leuconostoc mesenteroides*. No strains of *Leuconostoc dextranicum* are given and only one of *Leuconostoc citrovorum* which will be shown later to be *Leuconostoc mesenteroides*.

The present study is an attempt to define the limits of the genus and to examine the species within it. The last study of this small but important genus was made in 1931 by Hucker & Pederson. Since then Abd-el-Malek & Gibson (1948) have described a new species and Pederson & Albury (1955) have found strains of *Leuconostoc mesenteroides* which do not form slime from sucrose.

METHODS

The strains used during the work, together with the sources from which they were received, are listed in Table 1. During the preliminary work the cultures were grown in yeast glucose broth, but this was not a satisfactory medium. A new medium (YG citrate) was developed, containing 2% Evans peptone, 1% glucose, 0·5% NaCl, 0·6% Yeastrel, 1% Lemco, and 0·5% ammonium citrate, final pH 6·5. All the strains grew well in YG citrate. Some strains grew better in tomato glucose broth, but for other strains the tomato broth was found to be inhibitory. The cultures were grown at 30°C.

The stock cultures were maintained in Yeastrel (0·3%) glucose (1%) litmus milk in cold store, and were revived every 4–6 months. The methods described by Abd-el-

Table 1. *Source of cultures*

N.C.D.O. no.	Strain	Source	Classification (group)
From N.I.R.D. Collection			
527	S32	—	VI
528	DW22	—	VI
529	*B. bovis* (originally from S. Orla-Jensen)	—	IV
530	X22 (originally from C. S. Pederson: *Leuconostoc paracitrovorum*)	—	VI
531	D22	—	V
537	Mesenteroides	—	V
From C. S. McCleskey, Louisiana, through N.I.R.D. Collection			
538	158	—	VI
539	168	—	VI
540	548	—	VI
541	853	—	VI
542	—	—	VI
From E. I Garvie, N.I.R.D.			
543	LF2	Starter	I
545	DM9	Starter	I
546	25. C. 1	Milk	II
547	33. C. 5	Milk	VI
548	33. C. 8	Milk	VI
549	34. 22. 13	—	II
From J. Naylor, N.I.R.D.			
794	D67	Cheese	V
817	A110	Cheese	VI
876	DWF129	Cheese	VI
879	M63	Milk	III
880	SS24	Air of dairy	V
881	SS27	Air of dairy	V
882	SS29	Air of dairy	VI
883	SS46	Air of dairy	III
884	SS60*a*	Air of dairy	V
885	SS80W	Air of dairy	III
886	SS98	Air of dairy	III
887	SS136	Air of dairy	V
From M. E. Sharpe, N.I.R.D.			
769	Heller B	Langmolk	I
869	KR1	Sauerkraut	III
870	RM1	Rumen	III
871	SL19	Silage	III
872	SL48	Silage	III
873	SL50	Silage	III
From M. Zielinska, N.I.R.D.			
183	S1/14	—	V
From I. A. Bernstein, Michigan			
768	39	—	VI
From A. Møller Madsen, Hillerød, Denmark			
1033	BC1	—	I
1071	NZa	—	I
1085	F1	—	I
1086	d	—	III
From Th. E. Galesloot, Ede, Holland			
828	3405	—	I
829	3409	—	I
861	21	Cheese	V
862	22	Cheese	IV
863	30	Cheese	IV
864	44	Cheese	V
From T. Gibson, Edinburgh			
532	Kefir 1	Milk	II
533	Kefir 5	Milk	II
534	Kefir 15	Milk	II
535	Kefir 16	Milk	II
536	Kefir 18	Milk	II
550	Gibson	—	V
925	58	—	III
926	70	—	III
From A. C. Hayward, Birmingham			
955	C4/4/3	Cheese	III
956	C17/1	Cheese	II
957	G14	Cheese	III
958	N2/1	Cheese	III
959	S17	Milk	II
From D. Murray Smillie, Auchincruive			
705	D3	Starter	I
708	G3	Starter	I
From N.C.I.B., Teddington			
516	NCIB 8189	—	V
517	NCIB 3355	—	IV
518	NCIB 8013	—	VI
519	NCIB 8015	—	VI
520	NCIB 6109	—	VI
522	NCIB 8022	—	VI
523	NCIB 8023	—	VI
551	NCIB 8029	—	VI
552	NCIB 8172	—	VI
553	NCIB 8590	—	VI
From N. R. R. L. Peoria, U.S.A.			
796	B. 512F	—	VI
797	B. 523	—	VI
798	B. 742	—	VI
800	B. 1141	—	V
801	B. 1146	—	V
837	B. 614M	—	VI
838	B. 1117	—	VI
From A.T.C.C., Washington			
812	ATCC 8082	—	V
From N.C.T.C.			
824	NCTC 3739	—	IV
From C. S. Pederson, N.Y. State Agric. Exp. Sta., Geneva			
803	R 80 (slime−)	—	III
805	R83	—	III
807	535	—	VI
808	683	—	VI
809	C33	—	VI
810	R 80 (slime+)	—	V
811	6–67	—	VI

Malek & Gibson (1948) for the production of ammonia from arginine and for the production of gas from glucose were adopted.

The heat resistance of the strains was examined by inoculating 0·04 ml of a culture into YG citrate, heating for the required time at the temperature chosen, and then incubating the tubes for 3 days and examining for growth. The ability to grow at 37, 39 and 40 °C was tested in YG citrate and the cultures observed daily for growth for 7 days.

It was found that the leuconostoc species were easily inhibited and the ability to grow in low concentrations of methylene blue and NaCl were examined. The basal medium was yeast glucose broth, and growth after 2 days at 30 °C was considered positive, after 3 days slow. Incubation was not continued further.

The ability to ferment different carbon sources has long been the basis of the classification of the leuconostocs. The basal medium selected was 1·5 % peptone, 0·6 % Yeastrel, and 0·5 % NaCl. Brom-cresol purple was used as indicator. The carbon sources used were prepared as 2 % solutions sterilized by Seitz filtration and 0·5 ml was added aseptically to each 5 ml of broth. The cultures were incubated for 14 days.

Dextran production was examined on agar plates incubated at 22 °C for 5 days. The basal medium was 1 % tryptone, 0·5 % Yeastrel, 0·5 % K_2HPO_4, 0·5 % ammonium citrate and 5 % sucrose.

RESULTS

Definition of the genus Leuconostoc

The first problem was to find satisfactory limits for the genus, and these were based on the description given in *Bergey's Manual* (7th ed.) Breed, Murray & Smith (1957), pp. 506, 531. The genus is considered to consist of strains which are Gram-positive cocci which grow in chains but not in clumps. Often the cells are elongated and become egg-shaped. They are catalase negative and form gas from glucose. Abd-el-Malek & Gibson (1948) found that all their strains failed to form ammonia from arginine and this was also true of the strains included in the present study. When grown in litmus milk no strains reduce litmus, a few acidify and might curdle the milk, but the majority do not change this medium. No gas is formed in litmus milk. In the present study, strains which did not possess all the above characters were taken to be outside the genus.

On the fringe of any genus are strains which cause difficulties in classification, and the differences between the low-temperature gas-forming lactobacilli and the leuconostocs is small. In *Bergey's Manual* (1957, p. 531) it is suggested that the leuconostocs may become elongated into a rod form. If this is so then the division between the two genera no longer exists. Some rod forms were received but they were able to produce ammonia from arginine and so were considered to be lactobacilli. Not only may leuconostocs become elongated but lactobacilli may be very short rods. Niven, Castellani & Allanson (1949) have described a species which was later called *Lactobacillus viridescens*. This species fails to produce ammonia from arginine and can form polysaccharide from sucrose. Biochemically the difference between *Lactobacillus viridescens* and *Leuconostoc dextranicum* is slight. However, the former makes

inactive lactic acid and the latter laevo rotary, one is a rod, the other a coccus. A comparison of these species is given in Table 2. Orla-Jensen (1942) found some leuconostoc strains which produced inactive lactic acid: however, it is believed that these strains are not leuconostocs, and this point will be discussed later.

Table 2. *A comparison of* Lactobacillus viridescens (*Niven & Evans*) *and* Leuconostoc dextranicum (*Hucker & Pederson*)

	*Lactobacillus viridescens** (20 strains)	*Leuconostoc dextranicum*† (5 strains only examined)
CO_2 from glucose	+	+
Polysaccharide from sucrose	14+, 6−	+
Greening on blood agar	+	Not known
Final pH in glucose broth	4·6–4·8	4·7–4·9
Litmus milk	No change	No change
Growth at		
5 °C	+	+
40 °C	±	−
Growth in		
6·5 % NaCl	+	−
10·0 % NaCl	−	−
Hydrolysis of		
Na hippurate	−	Not known
Aesculin	−	−
Arginine	−	−
Gelatin	−	−
Starch	−	−
Fermentation of		
Xylose	−	−
Arabinose	−	−
Mannose	+	+
Fructose	+	+
Galactose	−	±
Lactose	−	±
Maltose	+	±
Sucrose	15+, 5−	±
Trehalose	4+, 16−	+
Raffinose	−	−
Inulin	−	−
Glycerol	−	−
Mannitol	−	−
Sorbitol	−	−
Lactic acid from glucose	DL	D (−)

* Taken from Niven *et al.* (1949). † Results obtained during current work.

Galesloot (1950) described strains of leuconostocs which were able to reduce litmus and curdle milk. However, four of his strains (861, 862, 863 and 864) were obtained and have apparently changed since first isolated. They all failed to change litmus milk. So far, therefore, no strains have been found which do not fit the description given above.

The separation of leuconostocs and pediococci

Most catalase negative cocci, i.e. the streptococci, produce marked changes in litmus milk in a few days. However, the pediococci are inactive ni litmus milk and although many strains are weakly catalase positive, catalase negative strains are also known. These catalase negative strains have been variously named streptococci and leuconostocs in the past, because pediococci until recently were hardly known outside the brewing industry. Two strains of cocci widely used in biochemical studies were shown some years ago to be misnamed leuconostocs. These strains, ATCC 8081 *Leuconostoc citrovorum* and ATCC 8042 *Leuconostoc mesenteroides*, are both pediococci Felton & Niven, 1953; Garvie, 1959). Both these strains are inactive in litmus milk, but both produce ammonia from arginine and do not form gas from glucose.

Recently, on examining the characters of the betacocci (leuconostocs) given by Orla-Jensen (1942), it was noticed that several of his strains, including nos. 43–47, produced inactive and not laevo-rotary lactic acid. These strains did not form slime and did not produce gas from glucose. Photographs of strains 44 and 46 are published (Plate XXIV) and they show the typical morphology of the pediococci. It is known that Orla-Jensen had cultures which are now called pediococci, because Mees (1934) examined two strains, nos. 1 and 2, of the tetracocci and concluded that they were *Pediococcus halophulis* and *Pediococcus pentoscens*, respectively. These strains were found by Orla-Jensen to be catalase positive, while those included with the betacocci were catalase negative. On p. 75 Orla-Jensen discusses the similarity in cultural conditions and morphology of the micrococci and betacocci—mentioning in particular the micrococci which have since been shown to be pediococci and the 'micrococci-like betacocci'. Furthermore, the strain of *Micrococcus freudenreichii* ATCC 8459, is listed as *Tetracoccus casei* (Orla-Jensen). It also is a catalase negative coccus and the results of a preliminary examination indicate that this strain should also be amongst the pediococci. Carbohydrate fermentations are of little assistance in separating the two genera and the results given by Orla-Jensen could equally well apply to pediococci or to leuconostocs.

Examination of cultures

The results of the detailed examination of the strains which fitted the definition of the genus leuconostoc given above are summarized in Table 3. The variations, particularly in groups 3–6, were considerable, but clear division into subgroups was not evident; rather, there appeared to be a continuous spectrum of characters. This was particularly noticeable in group 6, and cultures could be arranged with those attacking fewest carbohydrates at one end, and those attacking most at the other, the cultures in between giving a continuous picture rather than a division into intermediate types.

McCleskey, Faville & Barnett (1947) found four types of slime formation. These were correlated to some extent with serological findings (Leiva-Quiros & McCleskey, 1947). No such clear-cut divisions appeared in the strains examined here. It is felt that further work may reveal subdivisions or possibly that more species exist than are recognized at present.

Table 3. *The characteristics of the strains of* Leuconostocs *examined*

	Group I	Group II	Group IV	Group III	Group V	Group VI
No. of strains examined	10	9	5	17	16	31
Litmus milk	–	a or AC	–	–	–	a
Y.G.L.M.	A to ACr	A or AC	ACR	A, ACr, some G	AC, RG	AC, RG
Growth at °C						
37	–	+	4	14	+	28
39	–	+	–	13	10	21
45	–	–	–	–	–	–
Growth in						
3 % NaCl	1	5	3	16	+	29
6·5 % NaCl	–	–	–	12	–	4
Growth in 0·0005 % methylene blue	1	+ S	4	+	+	30
Dextran synthesis	–	–	+ Sl	–	+	+
Final pH in Y.D.B.	5·0	4·5 5·0	4·7 4·9	4·5 5·0	4·6 4·8	4·6 4·8
Diacetyl production in YG citrate	+	3	1	–	–	–
Resistance 55 °C for 15 min	–	+	2	8 Sl	12 Sl	24 Sl
Resistance 55 °C for 30 min	–	+	2	7 Sl	6 Sl	13 Sl
Acid from						
Arabinose	–	1	–	16	–	+
Xylose	–	–	–	5	+	24
Fructose	–	7	+	16	14	30
Glucose	+	+	+	+	+	+
Galactose	+	+	3	+	13	29
Mannose	–	8	+	+	15	30
Cellobiose	–	–	–	7 S	3	19
Lactose	+	+	2	5 S	10	16
Maltose	–	+	3	16	+	29
Sucrose	–	8	4	15	+	+
Trehalose	–	1	+	+	+	+
Melibiose	–	8	–	+	15	25
Raffinose	–	3	–	7	9	16
Dextrin	–	–	–	6	–	–
Amygdalin	–	–	2	12 S	5	15
Aesculin	–	–	–	7	8	29
Salicin	–	–	–	–	3	24
Arbutin	–	–	–	–	2	21
Mannitol	–	–	–	7 S	3	11
	L. cremoris	*L. lactis*	*L. dextranicum*	*L. mesenteroides*		

A = acid; a = slight acid; C = clot; R = fully reduced; r = partially reduced; G = gas; Sl = slight; S = slow; – = no strain active; + = all strains active; a figure denotes number of strains active.

All cultures grew at 10 °C. No cultures formed acid from sorbose, melizitose, rhamnose, inulin, starch, adonitol, dulcitol, erythritol, glycerol, inositol and sorbitol.

Group I (Leuconostoc cremoris)

The strains of group I are a homogeneous group which fail to grow at 37 °C and only form acid from glucose, galactose and lactose. Small amounts of gas are produced.

Orla-Jensen did not isolate any cultures similar to group I. However, Knudsen & Sørensen (1929) found strains which they called *Betacoccus cremoris* (X form), which are similar to group I strains. Hucker & Pederson (1931) described a non-sucrose fermenting species of leuconostoc. They stated that *Leuconostoc citrovorum* produces acid from fructose, glucose, galactose and lactose, but Table 15 of Hucker & Pederson shows that only a few strains form appreciable amounts of acid from fructose. Abd-el-Malek & Gibson found some strains which they considered to be *Streptococcus citrovorus* (Hammer) which fermented maltose.

The belief that Hammer (1920) isolated and examined strains similar to group 1 has become established, but has no basis in fact. Hammer was primarily interested in flavour production in butter starters and not in bacterial taxonomy. Some of Hammer's cultures undoubtedly were gas-forming cocci and should be called leuconostocs. Several strains of *Streptococcus citrovorus* and *Streptococcus paracitrovorus* were passed by Hammer to Hucker & Pederson. All formed slime from sucrose. Hammer himself stated that *Streptococcus citrovorus* could be separated from *Streptococcus paracitrovorus* by gas production. No gas was formed by *Streptococcus citrovorus*. It is not clear, therefore, why Hucker & Pederson should have decided that the non-sucrose fermenting *Leuconostoc* species which they had was the same as *Streptococcus citrovorus* (Hammer). Fortunately, two of Hammer's strains were deposited in the American Type Culture Collection; one of these, ATCC 8081, is now known to be *Pediococcus cerevisiae* and the other, ATCC 8082, was found to belong to group 5 (Table 4). *Pediococcus cerevisiae* forms diacetyl and does not form gas, and the description of *Streptococcus citrovorus* could apply to *Pediococcus cerevisiae*. Attempts to find the original cultures of *Streptococcus citrovorus* have not been successful. Therefore, the conclusion is drawn that, like Orla-Jensen, Hammer did not separate the pediococci and leuconostocs. This may have been unfortunate to the dairy industry which has used leuconostoc strains in butter starters and has overlooked the pediococci.

The name *Leuconostoc cremoris* is probably the correct name for cultures of group I, and it is used in preference to *Leuconostoc citrovorum* (Garvie, 1960).

Group II (Leuconostoc lactis)

The strains included in group II were first isolated and described by Abd-el-Malek & Gibson (1948). They were then given the name *Streptococcus kefir*. The authors recognized these strains as belonging to the gas producing, catalase negative cocci, but were of the opinion that they should be included along with the streptococci and not divided into a separate genus. *Streptococcus kefir* was originally described by Freudenreich (1897) and cultures believed to be the same were later more fully described by Evans (1918). Hucker & Pederson (1931) were of the opinion that the strains described by Evans were the sucrose, non-arabinose fermenting species of *Leuconostoc*, and considered *Streptococcus kefir* and *Leuconostoc dextranicum* to be synonyms. However, Evans's cultures fermented raffinose, salicin and mannitol, characters which could only place them with the *Leuconostoc mesenteroides* types. The one strain of *Streptococcus*

kefir examined by Hucker & Pederson, ATCC 341, was deposited in the Collection by Evans (Catalogue 4th ed., 1938) and was found to form slime from sucrose.

Group V strains are most closely related to those described by Evans (1918). Group II strains are different because they do not ferment mannitol or salicin and generally do not ferment raffinose. They do not form slime from sucrose. Group II strains being dissimilar from any of the previous conceptions of *Streptococcus kefir* should not be so called. All the strains of group II studied so far have come from milk and dairy products; also they are more active in fermenting lactose than the other species of *Leuconostoc*. It is suggested that a new name, *Leuconostoc lactis*, be used.

Table 4. *The characters of* Leuconostoc 517, 530 *and* 812 (*ATCC* 8082)

	Group IV 517	Group VI 530	Group V 812
Litmus milk	–	–	–
Y–G litmus milk	ACr	N.T.	ACRG
Growth at °C			
37	–	–	+
39	–	–	+
45	–	–	–
Growth in			
3 % NaCl	–	–	+
6·5 % NaCl	–	–	–
0·0005 % methylene blue	–	+	+
Final pH in Y.G.B.	4·7	N.T.	4·8
Diacetyl production	+	–	–
Resistance to 55 °C for			
15 min	–	–	Sl
30 min	–	–	Sl
Acid from			
Arabinose	–	+	–
Xylose	–	–	+
Fructose	+	+	+
Glucose	+	+	+
Galactose	±	+	+
Mannose	+	+	+
Cellobiose	–	–	–
Lactose	–	–	–
Maltose	±	+	+
Sucrose	–	+	+
Trehalose	+	+	+
Melibiose	–	+	+
Raffinose	–	–	–
Dextrin	–	–	–
Amygdalin	–	–	±
Aesculin	–	+	–
Salicin	–	–	–
Arbutin	–	–	–
Mannitol	–	–	–
Sorbitol	–	–	–
Dextran synthesis	+Sl	+	+

A = acid; C = clot; G = gas; R = fully reduced; r = partially reduced; Sl = slight.
N.T. = not tested.

Group III (Leuconostoc mesenteroides)

The third group of non-slime forming leuconostocs are more active in sugar fermentation than groups I and II. Pederson & Albury (1955) reported that strains could be induced to produce slime, and that strains with this property could lose it. There is no justification, therefore, in basing species differentiation on slime formation, and group III have been included as strains of *Leuconostoc mesenteroides*.

Group IV (Leuconostoc dextranicum)

This group was very small, consisting of only five strains. They all produced slime and failed to ferment either arabinose or xylose. They conform to the characters of *Leuconostoc dextranicum* (Hucker & Pederson). The differences in fermentation ability between groups II and IV is not great. The strains of group II can more easily attack lactose and generally fail to attack trehalose. The more important difference between groups II and IV is in heat resistance. Group IV fail to grow at 39 °C and are destroyed by heating to 55 °C for 15 min, while group II can grow at 39 °C and survive heating to 55 °C for 30 min. Very few strains which can be called *Leuconostoc dextranicum* were found and it may be a rare species. No strains so called are now listed in the catalogue of the American Type Culture Collection.

Group IV includes some interesting strains. Strain 529 was received as *Betacoccus bovis* and was reported to be one of Orla-Jensen's strains. This strain would confirm the opinion of Hucker & Pederson that *Leuconostoc dextranicum* and *Betacoccus bovis* were the same species.

Group IV also includes strain 517 received from the NCIB and listed as coming from C. S. Pederson, strain 22. Another strain, 530, was received as X 22 *Streptococcus paracitrovorus*, originally from C. S. Pederson. This strain fermented arabinose but not xylose and is included with group VI. The detailed results for these two cultures are given in Table 4. They may not have originated as the same culture, but both are now atypical of their groups and differ from each other.

Groups V and VI (Leuconostoc mesenteroides)

These groups have been separated on their ability to produce acid from pentoses. The distinction is not very great, and both groups are considered to be *Leuconostoc mesenteroides*. Davis (1950) suggested that the ability to hydrolyse salicin and aesculin was a characteristic of *Leuconostoc mesenteroides*. Many cultures in group VI would be considered *Leuconostoc mesenteroides* by these tests, but only a few in group V. No group III cultures attacked salicin. At present groups III, V and VI present a wide, rather unwieldy species, but on the results of the present tests no satisfactory subdivisions can be made.

REFERENCES

Abd-el-Malek, Y. & Gibson, T. (1948). *J. Dairy Res.* **15**, 233.
Breed, R. S., Murray, E. G. D. & Smith, N. R. (1957). *Bergey's Manual of Determinative Bacteriology*. London: Baillière Tindall and Cox.
Davis, J. G. (1950). *A Dictionary of Dairying*. London: Leonard Hill.
Evans, A. C. (1918). *J. agric. Res.* **13**, 235.
Felton, E. A. & Niven, C. F. (1953). *J. Bact.* **65**, 482.
Freudenreich, E. (1897). *Zbl. Bakt.* Abt. 2, **3**, 87.

GALESLOOT, TH. E. (1950). *Ned. melk-en Zuiveltijdschr.* **4**, 274.
GARVIE, E. I. (1959). *Nature, Lond.*, **183**, 1411.
GARVIE, E. I. (1960). In preparation.
HAMMER, B. W. (1920). *Res. Bull. Ia agric. Exp. Sta.* no. **63**.
HUCKER, J. G. & PEDERSON, C. S. (1931). *Zbl. Bakt.* Abt. 2, **85**, 65.
KNUDSEN, S. & SØRENSEN, A. (1929). *Den Kgl. Veterinaerog Landbohejskole. Aarsskrift* 64.
LEIVA-QUIROS, A. & MCCLESKEY, C. S. (1947). *J. Bact.* **54**, 709.
MCCLESKEY, C. S., FAVILLE, L. W. & BARNETT, R. O. (1947). *J. Bact.* **54**, 697.
MEES, R. H. (1934). *Onderzoekingen over de Biersarcina.* Diss. auf dem Lab. A. J. Kluyver, *Delft.*
NIVEN, C. F., CASTELLANI, A. G. & ALLANSON, V. (1949). *J. Bact.* **58**, 633.
ORLA-JENSEN, S. (1942). *The Lactic Acid Bacteria.* Copenhagen, Kommission Hos Ejnar Munksgaard.
PEDERSON, C. S. & ALBURY, M. N. (1955). *J. Bact.* **70**, 702.

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Variations in the rate of milk secretion in milking intervals of 2–24 hours

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Summary. The rates of secretion of milk, fat and solids-not-fat have been measured over milking intervals of 2–24 h in a series of experiments using thirty cows at various stages of lactation. To ensure that the results were not biased by a carryover of residual milk and fat the residual milk was removed after intravenous injections of 'Pitocin'. The biases that can be caused by previous interval effects and lactation trends were eliminated by the balanced Latin square design used.

Milk and solids-not-fat secretion appeared to be linear with duration of milking interval up to 16 h, but with longer intervals there was, in some experiments, a decline in the rate of secretion. There was considerable variation between cows in the decline in secretion rate with increasing milking interval and part of this variation was associated with differences in milk yield. In all experiments fat secretion appeared to be almost linear with time over a 24 h period.

After milking intervals of 20 and 24 h the rate of secretion of milk and solids-not-fat, but not of fat, was depressed for at least 16 h.

For many years it has been generally assumed that the rate of milk secretion into the udder progressively declines as milk accumulates in the udder and that this decline is appreciable even within the milking intervals that occur with twice-daily milking. This belief was based to a large extent on the experiment of Ragsdale, Turner & Brody (1924). It was also considered to be supported by the increase in milk yield that occurs when cows are milked three times a day rather than twice, as well as by the increases in yield believed to occur when cows are milked at equal rather than unequal milking intervals and also following more complete evacuation of the udder during milking.

However, it is only possible to draw conclusions about the rate of milk secretion from the usual type of milk-yield data if it is assumed that the milk obtained at

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a particular milking is equivalent to that secreted during the previous milking interval. This assumption was questioned by Krull (1905) and Bartlett (1929) and later by Johansson (1940) who was the first to demonstrate the considerable carry-over of milk and fat, in the form of residual milk, from one milking interval to the next and to appreciate its importance. He found that the carryover of residual fat was in direct proportion to the amount of milk in the udder before milking. This was confirmed by Bailey, Clough & Dodd (1954, 1955). Turner (1953, 1955*a*) has provided evidence that this is also true of residual milk.

Because of these variations in the quantity of retained residual milk and fat the yields obtained after a long interval following a short interval will be less than the amounts secreted. But where a short interval follows a long interval the yields obtained will be greater than that secreted.

A further factor likely to bias experiments on the rate of milk secretion was put forward by Turner (1953). He suggested that after milking intervals of more than 12 h the rate of secretion in the succeeding interval may be depressed. This preceding interval effect has been demonstrated by Bailey *et al.* (1955) and was mentioned by Elliott & Brumby (1955).

Thus in any experiment designed to measure the rate of milk secretion in which milking intervals of different lengths follow one another, biases due to residual milk carryover and preceding interval effects can be suspected. This makes unsound the indirect method of assessment of milk secretion rate from data collected in a twice-daily milking routine as was attempted by Bartlett (1929) and Edwards (1950). It also invalidates the conclusions from the experiments of Ragsdale *et al.* (1924) and Gasnier, Leroy & Maitrejean (1945) in which the experimental intervals followed a fixed night interval. Ragsdale *et al.* had concluded that there was a progressive decrease in milk secretion rate with increase in milking interval, and Gasnier *et al.* had concluded that this occurred after accumulation had proceeded for 6 h.

Eisenreich & Mennicke (1950) arranged their intervals in random order. Even so it is inevitable that the shorter intervals have longer preceding intervals and consequently both the residual carryover and preceding interval effects would be present. However, they concluded that both the milk and the fat secretion rates did not decline until the milking interval exceeded 15 h.

More recently direct measurements of the relationship between the quantity of milk secreted and the length of the milking interval have been made by Bailey *et al.* (1954, 1955), Turner (1955*b*) and Elliott & Brumby (1955). Bailey *et al.* concluded that the relationship was curvilinear over a period of 18 h though the deviation from linearity was negligible up to 12 h. Turner, covering a much wider range of milking intervals, found that 'secretion would not be significantly affected by length of interval up to at least 16 h'. Elliott & Brumby found that milk secretion rate 'shows no significant decline before 20–24 h'. The differences between these results may be due to inherent differences between the relatively small numbers of cows in the three samples, or to differences in the experimental methods of eliminating residual carry-over and preceding interval effects. Bailey *et al.* corrected for residual carryover effects by repeating each milking interval for at least five consecutive milkings. This did not, however, correct for any preceding interval effect. Turner, and Elliott & Brumby removed the residual milk by milking after an intravenous injection of an

oxytocin preparation. Providing the removal of residual milk is similarly effective over the complete range of milking intervals, this method would correct for the residual milk carryover by ensuring that at the start of each interval the quantity of residual milk in the udder would be a constant small amount. In addition, Turner, and Elliott & Brumby attempted to eliminate the preceding interval effect, Turner by a controlled randomization of the order of intervals, and Elliott & Brumby by inserting a 12 h interval before each experimental interval.

It would appear that in experiments where biases due to residual milk carryover and previous interval effects may be suspected, milk and fat secretion rates have declined as the length of the milking interval increased; that in experiments where residual milk differences are eliminated but the preceding interval effect is present, secretion rate has shown no marked decline until after 12 h; but where both are eliminated, secretion rate has appeared to be unaffected for 16–24 h.

These conclusions do not conflict with the results of the recent experiments carried out with monozygous twins on the effect of unequal milking intervals with twice-daily milking (Koshi & Petersen (1954); Turner (1955*c*); Hansson, Claesson & Brannang (1956); McMeekan (1956); McMeekan & Brumby (1956) and Hansson, Claesson, Brannang & Gustafsson (1958)). These experiments show either no difference in yield between milking at unequal and equal intervals or only a small reduction with unequal intervals. The increases in milk yield that occur with thrice-daily milking have been discussed by Hansson & Bonnier (1947). These may not be directly due to the differences in the amount of milk accumulating in the udder in 12 compared with 8 h and alternative explanations have been discussed by Elliott (1959*a*, *b*).

EXPERIMENTAL

The experiments described in this paper were those undertaken by Elliott & Brumby in New Zealand which have already been summarized (Elliott & Brumby, 1955) and those carried out by Elliott & Dodd in England.

The experiments were a series of short-term trials designed to measure the effect of the length of interval between milkings on milk secretion rate. Particular care was taken to avoid biases in the results due to the carryover of residual milk from one interval to the next, and the adverse effect of a long milking interval on the yield in the succeeding interval. Four experiments involving nine separate trials were carried out. The first two experiments were conducted in New Zealand at the Ruakura Animal Research Station and the remainder in England at the National Institute for Research in Dairying, Shinfield.

The object of the New Zealand experiments was to measure the effect of milking intervals of up to 24 h on milk and butterfat secretion rate, first, on six cows at three stages of lactation (Expt. 1), and then on a further twelve cows in mid-lactation (Expt. 2).

The purpose of the first Shinfield experiment (Expt. 3) was to investigate certain remaining problems regarding the experimental design. In the second Shinfield experiment (Expt. 4) the improved experimental design and techniques were used to measure the effect of the length of the interval on the secretion rate of higher yielding cows than those used in the earlier experiments.

The experimental design in all of the nine trials was a 6 × 6 balanced Latin square. With this design each of the six cows used in each trial was milked once after each of the six intervals. The order of the milking intervals was such that in each Latin square each interval followed every other interval once only and occurred only once in each position of the milking order. The basic design is summarized in Table 1. The rows of the square represent the animals, the columns represent the succession of the intervals, T_1–T_6 are the six milking intervals, and C is a constant intervening interval.

Table 1. *The balanced Latin square design used in all experiments. T_1 to T_6 are the six milking intervals, and C a constant intervening interval*

		Order											
		1		2		3		4		5		6	
Cows	A	C	T_1	C	T_2	C	T_3	C	T_4	C	T_5	C	T_6	C
	B	C	T_2	C	T_4	C	T_6	C	T_1	C	T_3	C	T_5	C
	C	C	T_3	C	T_6	C	T_2	C	T_5	C	T_1	C	T_4	C
	D	C	T_4	C	T_1	C	T_5	C	T_2	C	T_6	C	T_3	C
	E	C	T_5	C	T_3	C	T_1	C	T_6	C	T_4	C	T_2	C
	F	C	T_6	C	T_5	C	T_4	C	T_3	C	T_2	C	T_1	C

An attempt was made to eliminate the biases caused by residual milk carryover by removing the residual milk after each milking. The effect of the length of the preceding milking interval on the secretion during an interval was eliminated by the use of this particular Latin square design and also by the insertion of a constant interval between each of the experimental milking intervals. This constant intervening interval also enabled the previous interval effect of the various milking intervals to be measured. The results obtained from each experiment were analysed by the method outlined and discussed by Williams (1949).

To enable residual milk to be withdrawn the cows were injected with an oxytocic hormone (Parke Davis 'Pitocin') at every milking. Normal milking was carried out first, and in the first seven trials was followed by an intravenous injection of 10 i.u. 'Pitocin', followed by immediate re-milking. In the later trials this in turn was followed by a second injection of 10 i.u. of the hormone preparation and a further milking.

In the New Zealand experiments the 'Pitocin' was injected into the jugular vein. For the Shinfield experiments, a small-bore polythene tube was inserted into the subcutaneous abdominal (mammary) vein and held there by waterproof adhesive tape for the duration of the experiment. This technique proved successful, and provided a means of painless injection with no disturbance to the cow. Each dose of 'Pitocin' was made up to approximately 5 ml with normal saline to which sodium citrate had been added at the rate of 0·5 g/100 ml, to ensure that the tubes were kept free from clotted blood.

All milking and stripping was done by machine. The washing stimulus was employed at the beginning of milking.

The milk obtained by normal milking and the residual milk obtained after 'Pitocin' injection were weighed separately. In New Zealand the butterfat percentages of the

normal milk and the residual milk were separately assessed, but in England the combined total milk only was sampled to determine butterfat percentage, and in the later trials solids-not-fat percentage as well.

In New Zealand all experimental cows were at pasture day and night, and had no supplementary feeding. The four trials at Shinfield were carried out during the winter when the cows were in yards receiving a maintenance ration of hay and silage. Concentrates were fed at the rate of 4 lb/gal at milking time and were proportioned according to the length of the interval just ended. With the 20 and 24 h intervals, half the concentrate ration was fed to the cows half way through the interval.

Expt. 1. *The effect of milking intervals of* 4, 8, 12, 16, 20 *and* 24 *h on milk and butterfat secretion of six cows at three stages of lactation*

Six cows were used, four Jerseys and two Friesians. The average 12 h yields of milk were 17·4, 16·0 and 9·0 lb measured 62, 112 and 190 days after calving. The intervening constant interval in this experiment was 12 h and the same randomization of the square was used at all three stages of lactation. After each milking 10 i.u. 'Pitocin' was injected into the jugular vein to enable the residual milk to be removed.

Table 2. *A summary of the analyses of variance of Expts.* 1, 2, 3 *and* 4

(*a*) Effect of treatment intervals on milk secretion

		Treatment					
Expt. no.	Cows	Total	Linear	Quadratic	Order of milking	Carry-over	Error, %
1 (early lactation)	**	**	**	**	–	–	17
(mid-lactation)	**	**	**	*	–	–	17
(late lactation)	**	**	**	–	–	–	16
2 (group 1)	**	**	**	–	–	–	7
(group 2)	**	**	**	–	–	–	9
3*a*	**	**	**	–	–	–	10
b	**	**	**	–	–	–	10
4*a*	**	**	**	**	–	–	11
b	**	**	**	**	–	–	13

(*b*) Effect of length of treatment interval on the rate of secretion in the following interval (i.e. the intervening intervals)

1 (early lactation)	**	*	**	*	–	–	5
(mid-lactation)	**	–	**	–	–	–	6
(late lactation)	**	–	**	–	–	–	7
2 (group 1)	**	–	**	–	–	–	6
(group 2)	**	**	**	**	–	–	5
3*a*	**	–	–	–	–	–	3
4*a* (1st 8 h)	**	**	**	**	–	–	7
(2nd 8 h)	**	*	**	–	–	–	7

– Not significant ($P > 0{\cdot}05$).
* Significant when $0{\cdot}05 > P > 0{\cdot}01$.
** Significant when $P < 0{\cdot}01$.

Results

The results of this experiment are given in Figs. 1 and 2 and in Table 2 where the analysis of variance tables of all the experiments are summarized.

From Fig. 1 it may be seen that the rate of accumulation of milk within the udder during a 24 h period is curvilinear in early and mid-lactation and probably linear in late lactation, but in all instances there does not appear to be any measurable fall in the rate of secretion until the interval lengths exceed 12 h. This was confirmed by the analysis of variance (Table 2) which demonstrates a significant quadratic component of the treatment variance for the results obtained in early and mid-lactation only. To estimate the decline in secretion rate in 24 h the linear regression

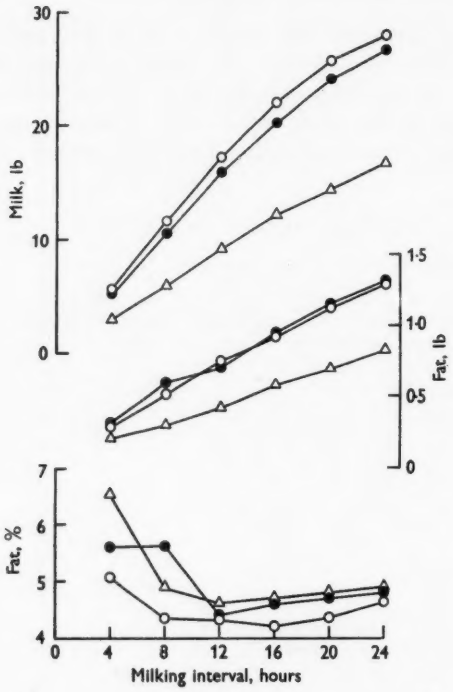

Fig. 1

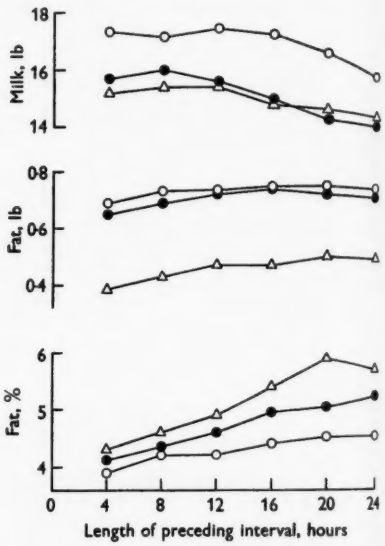

Fig. 2

Fig. 1. The quantities of milk and fat secreted and the changes in the fat content of the milk of six cows during milking intervals of 4–24 h (Expt. 1). ○, Early lactation; ●, mid-lactation; △, late lactation.

Fig. 2. The yields and fat contents of the milk secreted during the 12 h intervening intervals in Expt. 1. ○, Early lactation; ●, mid-lactation; △, late lactation.

lines of total milk secreted on the duration of the milking interval were calculated for the intervals of 4–16 h, and by extrapolation the yields of milk expected to be secreted in 20 and 24 h were calculated. The percentage reduction of the secretion in 20–24 h was calculated from the difference between the actual amount secreted in these intervals and the extrapolated values expressed as a percentage of the latter.

Calculated in this way the percentage reductions in the amount secreted in 20 h in early, mid- and late lactation were 7·9, 5·5 and 5·9 and in 24 h, 16·5, 12·4 and 9·2 (Table 3).

From Fig. 1 it appears that the quantity of butterfat secreted is linear with time over the full 24 h period at all three stages of lactation. The graph of the butterfat percentage shows an unexpected trend in that it falls rapidly with increase in interval from 4 to 12 h and then increases slowly. It is most unlikely that this trend is a true

Table 3. *The percentage reduction in the secretion of milk, fat and solids-not-fat in milking intervals of* 20 *and* 24 *h, found by extrapolation of the linear regression lines calculated for intervals of* 4–16 *h*

	Reduction in secretion of milk, %		Reduction in secretion of fat, %		Reduction in secretion of solids-not-fat, %	
Expt. no.	20 h	24 h	20 h	24 h	20 h	24 h
1 early lactation	7·9	16·5	—	—	—	—
1 mid-lactation	5·5	12·4	—	—	—	—
1 late lactation	5·9	9·2	—	—	—	—
2 (group 1)	1·5	5·1	—	—	—	—
2 (group 2)	0·5	4·2	—	—	—	—
4*a*	11·6	24·8	3·6	0·8	12·2	26·2
b	9·4	21·4	5·2	12·2	7·9	21·3

Table 4. *The estimated yield of milk, fat and solids-not-fat at a time interval of zero (i.e. the point where the regression line of the yields on milking intervals up to* 16 *h cuts the y-axis)*

	Milk		Fat		Solids-not-fat	
Expt.	Quantity, lb	Time equivalent, min	Quantity, lb	Time equivalent, min	Quantity, lb	Time equivalent, min
1 early lactation	0·5	22	0·09	108	—	—
1 mid-lactation	0·8	39	0·13	156	—	—
1 late lactation	0·1	8	0·05	94	—	—
2 (group 1)*	0·1	6	0·06	100	—	—
2 (group 2)	0·1	6	0·06	100	—	—
3*a*	−0·4	−13	0·01	8	–	–
b	0·3	11	<0·01	<8	—	—
4*a*	0·4	15	0·05	23	0·03	33
b	<0·1	<4	0·03	14	<0·01	<10

* Regression calculated for milking interval of up to 12 h.

reflexion of change in the rate of butterfat secretion relative to milk secretion, and the more likely explanation is that in spite of the use of 'Pitocin' all the residual fat was not removed at each milking, and as a result there was a carryover of fat secreted in the constant 12 h intervals to the following intervals. This residual carryover of milk of high butterfat content appears to have been sufficient to increase greatly the fat percentage of the small amount of milk secreted during the 4 h intervals, but was insufficient to affect materially the fat content of the large quantities of milk secreted in the longer intervals. The belief that there was a carryover of residual milk is supported by the fact that the linear regression lines of milk and butterfat secretion (y) on the duration of the milking intervals (x), calculated for intervals of 4–16 h, cuts

the y-axis appreciably above the intersection with the x-axis and not near the origin as might be expected. Thus when x is zero the values of y in early, mid- and late lactation were 0·5, 0·8 and 0·1 lb of milk—quantities which would have been secreted in 22, 39 and 8 min, respectively. For butterfat, the values were 0·09, 0·13 and 0·05 lb which would require 108, 156 and 94 min to be secreted (Table 4).

The pattern of the secretion of milk and fat during the intervening 12 h intervals may be seen from Fig. 2. The smaller amounts of milk secreted in the 12 h intervals that followed milking intervals exceeding 12 h in length are apparent. The effect is significant ($P < 0·05$). The variations in the fat percentage are very probably spurious and due to the carryover of residual fat as previously discussed.

Expt. 2. The effect of milking intervals of 4, 8, 12, 16, 20 *and* 24 *h on milk and butterfat secretion of twelve cows in mid-lactation*

The object of this experiment was to extend the investigation to a further twelve Jersey cows in mid-lactation. The average 12 h milk yield of these cows was 11·9 lb and the average interval between calving and the start of the experiment 135 days.

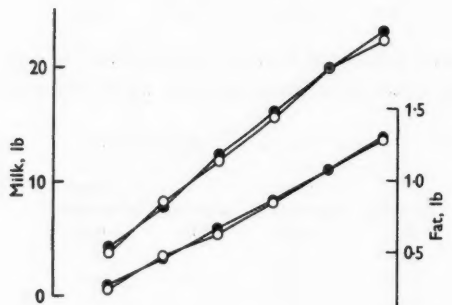

Fig. 3

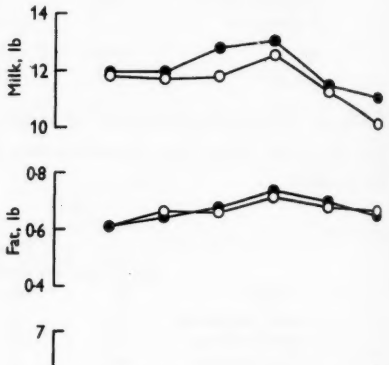

Fig. 4

Fig. 3. The quantities of milk and fat secreted and the changes in the fat content of the milk of twelve cows during milking intervals of 4–24 h (Expt. 2). ●, Group 1; ○, group 2.

Fig. 4. The yields and fat contents of the milk secreted during the 12-hour intervening intervals in Expt. 2. ●, Group 1; ○, group 2.

The design of the experiment, and the milking and the injection procedures were the same as in Expt. 1. The two groups of six cows (groups 1 and 2) were treated exactly alike.

Results

The results of this experiment, given in Figs. 3 and 4 and Tables 2, 3 and 4 are similar to those of Expt. 1 except that the rates of secretion of both milk and butterfat are linear with time over the whole 24 h period. The trends in butterfat percentage are also similar to those in Expt. 1 probably because of incomplete removal of residual fat.

Using the methods described above the percentage reductions in the 20 and 24 h intervals were found to be 1·5 and 5·1 for group 1 and 0·5 and 4·2 for group 2. The point at which the regression lines of milk and fat secretion on length of the milking interval cut the y-axis is the same for both trials, namely 0·1 lb of milk equivalent to 6 min secretion, and 0·06 lb of fat, equivalent to 100 min secretion.

In spite of the fact that the rate of milk secretion did not decline during the course of a milking interval of 24 h, it appears that milking intervals of 20 and 24 h reduced the secretion of milk in the succeeding 12 h intervals (Fig. 4).

Expt. 3. *The effect of milking intervals of* 2, 4, 6, 8, 10 *and* 12 *h on milk and butterfat secretion using two different experimental methods*

The analyses of Expts. 1 and 2 indicated the strong possibility that the removal of residual milk was incomplete and as a result the rates of secretion, particularly of fat, were biased during the short intervals. To investigate this hypothesis two designs were used in Expt. 3. The first (3*a*) was identical with that used for Expts. 1 and 2 where each of the experimental milking intervals followed a 12 h interval. The second (3*b*) had the same Latin square design, but each interval was repeated three times at consecutive milkings and there were no intervening constant intervals. This experiment was limited to milking intervals of up to 12 h because biases caused by residual carryover are greater in the short intervals, and previous interval effects appear to be negligible at intervals of less than 12 h. It was expected that the fat percentages of the milk obtained after the intervals in Expt. 3*a* would show the same trends as in Expts. 1 and 2, because the residual carryover from the 12 h constant interval would have a greater effect on the fat percentage of the milk secreted in the short intervals.

If the removal of residual milk from the cows used on Expt. 3*a* was incomplete, then it follows that it would also be incomplete in Expt. 3*b*. However, in the latter the design of the experiment should eliminate any bias of the measurement of rate of secretion because residual milk is proportional to milk yield. Each interval was repeated consecutively three times and for the last interval the amount of residual milk and fat in the udder at the start of the interval was expected to be the same as that left at the end. As a result the yield of milk obtained should be equivalent to the true amount secreted (cf. Bailey *et al.* 1955).

There was one other change which subsequently appeared to be important. The 'Pitocin' was injected through a mammary vein cannula and as a result was painless, the cows being unaware that any injection had been made.

Results

The rates of milk and butterfat secretion in Expts. 3*a* and *b* were both linear with time over the 12 h range and the fat percentages were nearly constant not showing the variations found in Expts. 1 and 2. This is shown in Fig. 5 and Table 2. Fig. 6 indicates that milking intervals of up to 12 h do not adversely affect the secretion in the subsequent intervals.

This experiment demonstrates that, providing there is a complete elimination of the residual carryover of milk and fat (Expt. 3*b*), the fat content of the milk obtained after the various intervals does not show large variations, and the regression of the quantity of milk and fat secreted on the duration of the milking interval almost passes through the origin (Table 4). However, the experiment failed in its main

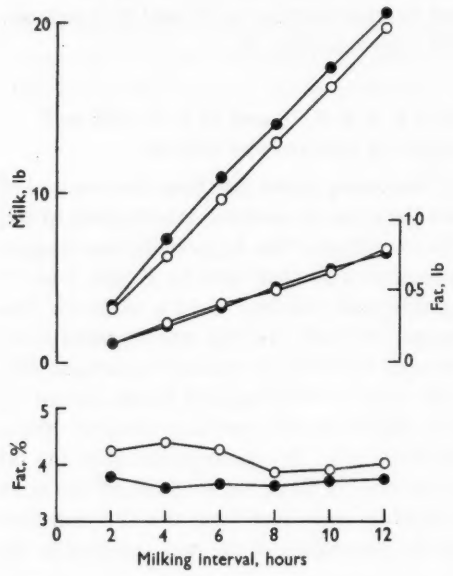

Fig. 5

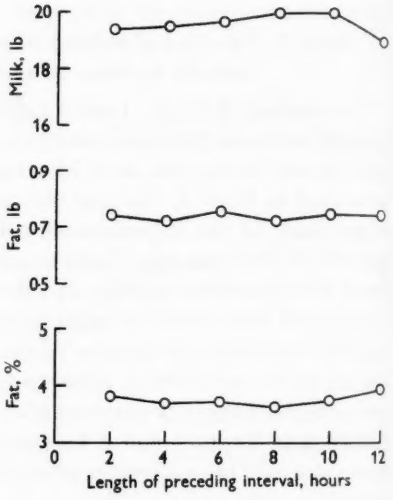

Fig. 6

Fig. 5. The quantity of milk and fat and the fat content of the milk secreted in milking intervals of 2, 4, 6, 8, 10 and 12 h (Expts. 3*a*, *b*). ○, Expt. 3*a*; ●, Expt. 3*b*.

Fig. 6. The milk and fat secreted in the 12 h intervening intervals in Expt. 3*a*.

objective of demonstrating differences between the results obtained by the two experimental methods. The reasons why the variations in the fat percentage of the milk obtained at the various intervals in Expt 3*a* (Fig. 5) were much less than in Expts. 1 and 2 can only be conjectured. It seems possible that the changed method of injection of 'Pitocin' might be the cause. Injection through an established plastic cannula causes no disturbance or pain, unlike the direct injection method used in Expts. 1 and 2, which would be likely to induce an involuntary release of adrenalin making the removal of residual milk less effective.

Expt. 4. *The effect of milking intervals of* 4, 8, 12, 16, 20 *and* 24 *h on milk, butterfat and solids-not-fat secretion using the two experimental designs of Expt.* 3

The objects of this experiment were to obtain further information on the effect of milking intervals of up to 24 h on higher yielding cows than were used in Expts. 1 and 2, to measure the changes in the solids-not-fat content of the milk obtained after these intervals, and to investigate the differences in results obtained from the two experimental designs.

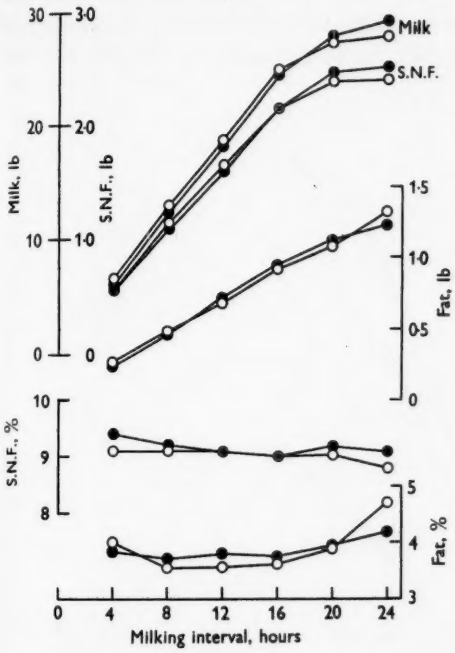

Fig. 7

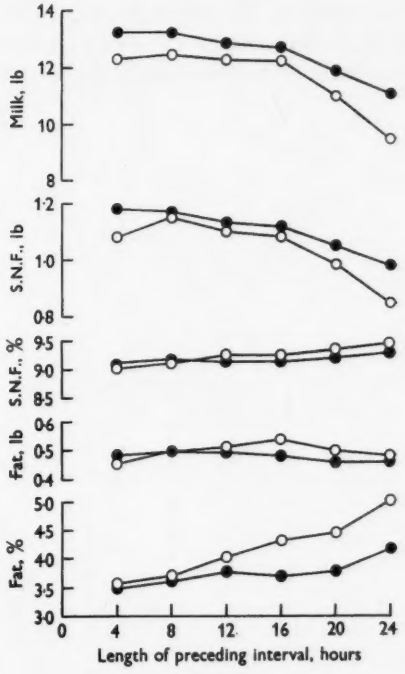

Fig. 8

Fig. 7. The quantities of milk, fat, and solids-not-fat secreted in milking intervals of 4, 8, 12, 16, 20 and 24 h using two experimental designs (Expts. 4*a*, *b*). The solids-not-fat is expressed as a percentage of the fat-free milk, ○, Expt. 4*a*; ●, Expt. 4*b*.

Fig. 8. The quantities of milk, fat, and solids-not-fat secreted in the intervening 8 h intervals in Expt. 4*a*. The solids-not-fat is expressed as a percentage of the fat-free milk. ○, First 8 h interval; ●, second 8 h interval.

Eleven of the twelve cows used were those from Expts. 3*a* and *b* and the experiment followed almost immediately after 3.

The two designs were basically the same as for Expts. 3*a* and *b* but the intervals were 4, 8, 12, 16, 20 and 24 h and in design *a* the intervening interval was changed from one of 12 h to two of 8 h. Design 3*b* was unchanged with three consecutive similar intervals for each treatment. This change was made because the previous interval effect of a 24 h interval had seemed to require more than 12 h before it was expended.

In this experiment two separate injections each of 10 i.u. 'Pitocin' were made at the end of every interval, each being followed by milking (cf. Johansson, 1940). The injection was by mammary vein canula. The total solids content of the milk was estimated gravimetrically, the fat content by the Gerber method, and the solids-not-fat content by subtraction.

Results

The mean effects of the treatments on milk secretion may be seen in Figs. 7 and 8 and Table 2. The solids-not-fat content is expressed as a percentage of the fat-free milk to eliminate the effect of variation in fat content on the solids-not-fat content of the milk. From the data it may be seen that the fat secretion was linear with time

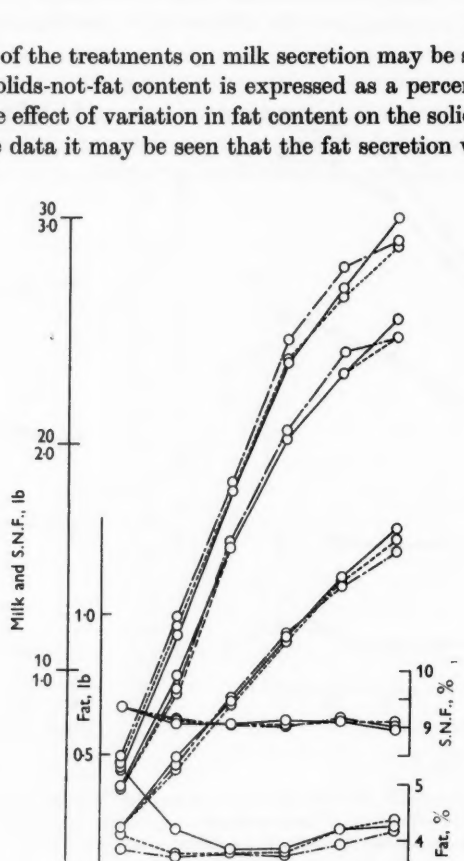

Fig. 9. The quantities of milk, fat and solids-not-fat secreted in all the milking intervals of Expt. 4*b*, where each interval was repeated three times at successive intervals. The solids-not-fat is expressed as a percentage of the fat-free milk. O—O, First milking interval; O - - - O, second milking interval; O – · – · O, third milking interval.

over the whole 24 h but that of the total milk and the solids-not-fat, though approximately linear up to intervals of 16 h, declined considerably in the 20 and 24 h intervals. The fat percentage of the milk increased from about 3·5 to 4·5 as the intervals increased from 12 to 24 h.

The percentage reductions in secretion at 20 and 24 h are much greater for milk in Expts. 4*a* and *b* than in previous experiments (Table 3). The success of both the treatments in removing the biases caused by the residual carryover of milk and fat may be seen from Table 4. The regression lines of milk, solids-not-fat and fat yield cut the *y*-axis almost at the origin, with design 4*b* the more accurate in this respect.

In Expt. 4*a* there were two consecutive 8 h intervals between the treatment intervals, and the data from the intervening intervals are given in Fig. 8. The previous interval effect on milk secretion was again evident and the depression in secretion in an 8 h interval was approximately 25 % when this interval followed a 24 h interval. It is interesting to observe that milk secretion in the second of the two 8 h intervals was depressed by about 10 % when the two 8 h intervals followed one of 24 h. The effect of the length of the previous interval on solids-not-fat secretion was also demonstrated.

The milk secreted in the three consecutive similar intervals is shown in Fig. 9. The graphs of the total secretion of milk, fat and solids-not-fat at the first, second and third milking for each treatment interval are very similar. In each case the secretion in the first of the three 24 h intervals was greater than the yield in the following two 24 h intervals. However, the 1½ lb or 5 % drop in the milk yield of a 24 h interval following another 24 h interval was small compared with the 3 lb of milk (25 %) drop in secretion when an 8 h interval followed a 24 h interval (see Fig. 8). The carryover of residual fat from the longer intervals to the following 4 h intervals was evident from the differences between the fat content of the milk obtained at the three succeeding 4 h intervals.

The percentage reductions in secretion during 20 and 24 h intervals determined by the method previously described are given in Table 3.

DISCUSSION

The results of these experiments indicate that when biases due to residual milk carryover and previous interval effects are eliminated, the rate of secretion of milk is virtually linear for 16 h and, thereafter it may decline. Of the six trials in which the secretion was measured during intervals up to 24 h (Expt. 1 early, mid- and late lactation, Expt. 2 groups 1 and 2, and Expt. 4*a*) there was a significant decline in the rate of secretion in three of the trials. In all trials the rate of fat secretion was apparently linear for 24 h, while in Expt. 4*a* it was shown that the secretion of solids-not-fat declined rapidly once the milking interval exceeded 16 h.

Under normal milking conditions where residual milk is not removed from the udder there will always be a quantity of milk in the udder at the start of each milking interval. This residual milk is equivalent to the amount secreted in 1–3 h (Turner, 1955*b*). Thus it appears that under these conditions the relationship between secretion and time can be regarded as almost linear for at least 13 h and in some cases as long as 23 h.

No reliable estimates may be made from these data of the effect of repeated long-milking intervals. Both Turner (1955*b*) and Claesson, Hansson, Gustafsson & Brannang (1959) have shown that milking once a day over a period has a much greater effect on milk secretion than when it is applied for only 1 or 2 days.

The results presented refer to means of groups of cows. However, it will be seen from Table 2 that there were significant differences between cows, and Fig. 10 illustrates the marked differences in the curvilinearity of the rate of milk secretion graphs of individual cows.

As an estimate of the curvilinearity of the relationship between total milk secretion and duration of milking interval up to intervals of 24 h the quadratic coefficient was calculated for the twenty-four cows used in Expts. 1, 2 and 4. Only one of the three sets of data collected on each cow in Expt. 1 was used and the regression line of the curvilinearity (y) on milk yield in 12 h (x_1) was calculated (Fig. 11). After eliminating

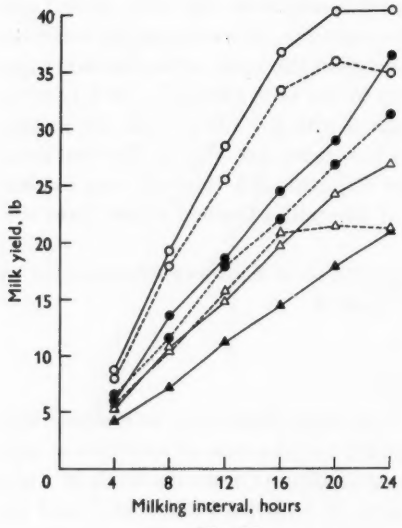

Fig. 10

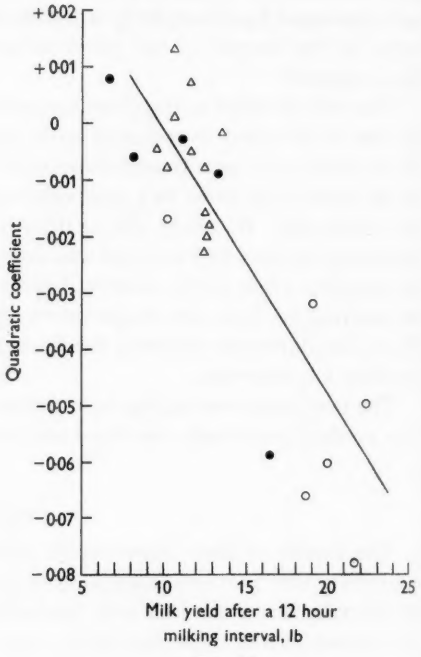

Fig. 11

Fig. 10. The accumulation of milk in the udders of seven of the cows used in Expts. 1, 2 and 4 in intervals of 4–24 h.

Fig. 11. The relationship between the curvilinearity of the regression of milk secretion on duration of milking interval and milk yield. The curvilinearity is measured as the quadratic coefficient, and milk yield by the quantity secreted in 12 h. ●, Expt. 1; △, Expt. 2; ○, Expt. 3.

the variation that could be attributed to differences between the various experiments the partial correlation coefficient of the quadratic coefficients (y) with the 12 h milk yield (x_1), independent of stage in lactation (x_2), was found to be $-0{\cdot}623$ ($p < 0{\cdot}01$), while that with stage of lactation independent of yield is $-0{\cdot}270$ ($p > 0{\cdot}05$). From this analysis and Figs. 1 and 11 it would appear that one of the main factors affecting the decline in the rate of milk secretion with increasing milking interval is the yield level of the cows, and that this applies both within and between cows. It is, however, clear from Fig. 10 that other factors apart from milk yield are important in this

respect. Our results do not support the conclusion of Turner (1955*b*) that in general 'any given long interval causes similar proportional reduction of yield in late as in early lactation'. That we did not find a relationship between the curvilinearity and stage of lactation independent of yield level may be due to the very small differences between the stages of lactation of the animals used.

The results indicate that in Expts. 1 and 2 the removal of residual milk was not complete, and as a result the fat yields at least in the short intervals cannot be regarded as accurate estimates of the rate of fat secretion. From Expts. 3 and 4, particularly 3*b* and 4*b* where the results are not biased by residual milk carryover, it appears that the rate of fat secretion is inhibited less by the longer intervals than is milk secretion. In fact secretion of fat appears to be linear over the full 24 h period, and as a result the milk obtained after the longer milking intervals has the higher fat content confirming the earlier results of Bailey *et al.* (1955). The rate of secretion of the solids-not-fat declines with increasing interval at a rate at least as great as the total milk (Fig. 7).

There is a further possible explanation of the changes found in milk composition. This is that the rate of secretion of all the constituents of milk is linear with time up to milking intervals of at least 24 h, but that fractions are selectively reabsorbed into the blood during storage (Hansson, Dassat & Claesson, 1954). Thus, Fig. 7 can be interpreted as indicating that water and some of the constituents of solids-not-fat are reabsorbed during storage, while fat is not. Although it is probable that some reabsorption does take place during long intervals, it is unlikely that it is the cause of the observed changes in milk composition. The fact that an interval of 20 or 24 h caused large changes in the composition of the milk obtained after the succeeding short intervals, when reabsorption would be very slight, suggests that the changes in milk composition were mainly due to changes in the relative rates of secretion of the constituents (see Fig. 8).

Fig. 8 indicates that the secretion of fat does not appear to be affected by long previous milking intervals. Because the total milk is reduced the fat content of milk secreted in a short interval following a long interval is considerably increased. It is possible that this change in fat content in the relatively short intervals is due to the carryover of residual fat from the long intervals, though the results of Expt. 4*b* suggest that this is unlikely (Fig. 9).

Because the lengths of the intervening intervals were 12 h in the New Zealand experiments and 8 h in those carried out in England, it is not easy to relate the reduction in yield following a long milking interval with other factors. It appears from an examination of the results from individual cows that the depressing effect of a long milking interval on the milk secreted in the following interval is to some degree related to the average daily yield. It is also evident from Fig. 8 that milk secretion remains depressed for at least 16 h after the end of a long interval.

The results obtained from all the experiments indicate that the balanced Latin square design and the experimental techniques used were very suitable for this type of experiment. It would also appear that these experimental methods could be employed to measure the effects of various treatments on the relative rates of secretion of the constituents of milk. The insertion and establishment of mammary vein cannulae is relatively easy and makes the removal of residual milk more effective.

We are indebted to Dr C. P. McMeekan, Director of the Ruakura Animal Research Station, N.Z. and to Professor H. D. Kay, Director of the National Institute for Research in Dairying, England, for help and encouragement in this work. We would like to thank the Animal Health Trust for funds which enabled the work to be continued in England, Dr S. J. Rowland in whose laboratory the chemical analyses of milk were carried out, and Mr J. P. L. Gibbons, Mr T. K. Griffin, Mr J. Hughes and Mr H. Pos for help in the experiments. In addition we acknowledge the help of Mr C. P. Cox in the interpretation of the statistical data and of Dr F. Yates of Rothamsted Experimental Station in whose department most of the statistical computations were carried out.

REFERENCES

BAILEY, G. L., CLOUGH, P. A. & DODD, F. H. (1954). *Nature, Lond.*, **173**, 404.
BAILEY, G. L., CLOUGH, P. A. & DODD, F. H. (1955). *J. Dairy Res.* **22**, 22.
BARTLETT, S. (1929). *J. agric. Sci.* **29**, 36.
CLAESSON, O., HANSSON, A., GUSTAFSSON, N. & BRANNANG, E. (1959). *Acta agric. scand.* **9**, 38.
EDWARDS, J. (1950). *J. agric. Sci.* **40**, 100.
EISENREICH, L. & MENNICKE, U. (1950). *Milchwissenschaft*, **5**, 140.
ELLIOTT, G. M. (1959*a*). Ph.D. Thesis, University of Reading, England.
ELLIOTT, G. M. (1959*b*). *Dairy Sci. Abstr.* **21**, 481.
ELLIOTT, G. M. & BRUMBY, P. J. (1955). *Nature, Lond.*, **176**, 305.
GASNIER, M., LEROY, A. M. & MAITREJEAN, M. (1945). *Ann. Agron.* Paris, **15**, 434.
HANSSON, A. & BONNIER, G. (1947). *Acta agric. suec.* **2**, 311.
HANSSON, A., CLAESSON, O. & BRANNANG, E. (1956). *Proc. IXth. Int. Dairy Congr.* **1**, 194.
HANSSON, A., CLAESSON, O., BRANNANG, E. & GUSTAFSSON, N. (1958). *Acta agric. scand.* **8**, 296.
HANSSON, A., DASSAT, P. & CLAESSON, O. (1954). *Riv. Zootec., Milano*, **27**, 316.
JOHANSSON, I. (1940). Copy of a paper prepared for the International Dairy Congress to have been held in Vienna, 1940.
KOSHI, J. H. & PETERSEN, W. E. (1954). *J. Dairy Sci.* **27**, 673.
KRULL, F. (1905). Quoted by Johansson (1940).
MCMEEKAN, C. P. (1956). *Proc. Ruakura Fmrs' Conf. Hamilton, N.Z.*, p. 199.
MCMEEKAN, C. P. & BRUMBY, P. J. (1956). *Nature, Lond.*, **178**, 799.
RAGSDALE, A. C., TURNER, C. W. & BRODY, S. (1924). *J. Dairy Sci.* **7**, 239.
TURNER, H. G. (1953). *Aust. J. agric. Res.* **4**, 118.
TURNER, H. G. (1955*a*). *Aust. J. agric. Res.* **6**, 514.
TURNER, H. G. (1955*b*). *Aust. J. agric. Res.* **6**, 145.
TURNER, H. G. (1955*c*). *Aust. J. agric. Res.* **6**, 530.
WILLIAMS, E. J. (1949). *Aust. J. Sci. Res.* A**2**, 149.

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Reviews of the progress of dairy science

Section D. Nutritive value of milk and milk products

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(*Received* 15 *February* 1960)

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PART 1

INTRODUCTION

This section reviews a selection of papers which have reached New Zealand since the last review (1) was completed in January, 1958. As in the past, it covers general aspects of the nutritive value of milk for man and then deals more specifically with the nutritive value of milk fat and the fat-soluble vitamins in milk.

It would seem that once again we must consider the period under review as one of consolidation of knowledge rather than as one productive of any very significant advances. At the outset, however, passing reference might be made to two topics which although not directly related to the field under review have been productive of a very extensive literature and are of considerable interest, and indeed concern, to those nutritionists whose interests extend to maintaining and increasing the present consumption of milk and its products.

One of these, the possible implication of animal fats in the increasing incidence of coronary diseases, was referred to in the last review. Criticism of the number of

hypotheses and the few scientifically established facts would seem to have been justified, and there appears at the present time a clearer realization of the complexity of the problem and greater restraint in implicating fats in general and milk fat in particular. Indeed, with increasing attention being paid to the essential fatty acids in the diet and their protective role, it is possible that milk fat, along with certain other fats, may be found to play an important part in providing protection against atherosclerotic conditions. This suggestion is advanced by Halden (2) in a review of recent work and is also in line with the conclusions of other workers (3,4,5). The main weight of scientific evidence would now seem to be against any drastic change in human dietaries at the present time. Nevertheless, the general public is still being treated to a host of conflicting recommendations for the prevention of coronary heart diseases and these have drawn authoritative comment from the Food and Nutrition Board of the National Research Council (American) in its recent revision of the Recommended Dietary Allowances (6). After discussing the general problem, the report concludes that, '...It is not yet possible to state definitely a reasonable allowance for fat in the diet or to indicate the characteristics of a fatty acid mixture most favourable for the support of health. A diet selected from a wide variety of foodstuffs, both vegetable and animal, is most likely to maintain good health.' Further indications of the present status of knowledge of the various factors in the etiology of atherosclerotic heart diseases may be gained from a number of recent reviews (e.g. (7,8,9,10)).

The other topic, the contamination of milk and its products with radio-active isotopes, presents a more disquieting picture, not that there is any very real problem at the present time but rather that the increasing rate of contamination may well outstrip our knowledge of its hazards. This growing concern throughout the world is reflected in the very rapidly developing literature in this field. Nutritional hazards associated with radio-active contamination have been the subject of a recent Nutrition Society Symposium (11) in which our lack of the knowledge necessary to make any accurate estimate of the dangers both now and in the future was stressed, and a policy of more research work and less talk was advocated. Apart from investigations relating to the Windscale accident in 1957 (e.g. (12)) which illustrate the risk of a sudden severe contamination (in this case with ^{131}I) of dairy products to the extent that they were rendered unsafe for human consumption, most work on milk has centred round its ^{90}Sr content. This of course stems from the important place of milk as a source of calcium in the human diet and, as a result, the fact that the levels of ^{90}Sr in milk and its products virtually control the amounts of this isotope deposited in human bones. Literature covering the movement of ^{90}Sr from the radio-active fall-out through the soil, into the pastures, and eventually into milk is not relevant to this review, but it is clear that the level of ^{90}Sr in milk has increased over recent years and this increase, if unchecked, must eventually constitute a serious nutritional hazard (e.g. (13,14,15,16,17)). Of a number of factors which will, however, contribute some useful degree of protection, the ability of the cow to discriminate against strontium in milk production is probably the most significant. It would appear also that the removal of ^{90}Sr from contaminated milk is feasible, even on a commercial scale, using ion exchange resins (18,19). Natural causes of radio-activity in milk and the effects of nuclear fall-out have been fully reviewed by McWeeny (20).

NUTRITIVE VALUE OF MILK FOR MAN

Reports for milk production during 1957 and 1958 indicate that the post-war upward trend is still being maintained. The estimated world increase for 1957 over the previous year was 2% (21) with a further increase of 1% in 1958 (22). Associated with these increases have been slight changes in the general pattern of milk utilization. The year 1957, with an increase of 4% over the 1956 figure, set a post-war record for butter production (23) and there was a further increase of 3% in 1958 (24). Corresponding increases in cheese production were 4% in 1957 and 1% in 1958. From the many reviews of production and utilization of dairy products throughout the world, those reported in (25,26,27) supply the type of information with which the nutritionist is usually concerned.

The XVth International Dairy Congress was held in London during the period under review, but on this occasion the nutritive value of milk and its products would seem to have attracted less attention than hitherto. Nevertheless, the reports of the Congress contain much of at least indirect nutritional interest and a few strictly nutritional contributions are referred to later.

(*a*) *Adult nutrition*

Recent work on the nutritive value of milk and its products has been reviewed by several workers. Dědek & Lukáš (28) discuss the place of milk in human nutrition and make special reference to the therapeutic uses of certain special milks. They also discuss the problem of arteriosclerosis in relation to the consumption of dairy products. Milk and its uses in medicine, dietetics and nutrition have also been fully reviewed (29,30), and a number of the reviews and contributed papers presented at the Heinz International Symposium on Nutrition (31) have a direct bearing on the place of milk in both adult and infant nutrition.

de Haas (32), in a review on the nutritive value of milk protein and milk fat, points out that the value of the former has tended to be neglected, whereas that of the latter has been over-emphasized. This point of view will, of course, meet with general agreement. It is unfortunate that simple and convenient methods for estimating fat but not protein have long been available, and largely as a result of this fat has become, irrespective of the purpose for which the milk is to be used, the basis of payment and the criterion of herd improvement work. The current, but rather belated, attention to rapid methods for estimating proteins in milk is naturally of considerable nutritional interest.

An excellent review by Kon (33) covers the composition and nutritive value of milk, treated milks and milk products, the place of milk in national diets, and the use of milk and its products in child feeding schemes. Another very valuable contribution by the same author (34) discusses the influence of heat treatment and light on the composition and quality of milk. It seems relevant to repeat here a number of general points made by Kon on the place of milk in the diet and on the controversial subject of the effect of processing on the nutritive value of milk and its products; they are largely self-evident but need frequent restatement, since they tend to be lost sight of in some workers' enthusiasm for certain pet theories and ideas. In the first place, it detracts in no way from the importance of milk and of its contribution to

hypotheses and the few scientifically established facts would seem to have been justified, and there appears at the present time a clearer realization of the complexity of the problem and greater restraint in implicating fats in general and milk fat in particular. Indeed, with increasing attention being paid to the essential fatty acids in the diet and their protective role, it is possible that milk fat, along with certain other fats, may be found to play an important part in providing protection against atherosclerotic conditions. This suggestion is advanced by Halden (2) in a review of recent work and is also in line with the conclusions of other workers (3,4,5). The main weight of scientific evidence would now seem to be against any drastic change in human dietaries at the present time. Nevertheless, the general public is still being treated to a host of conflicting recommendations for the prevention of coronary heart diseases and these have drawn authoritative comment from the Food and Nutrition Board of the National Research Council (American) in its recent revision of the Recommended Dietary Allowances (6). After discussing the general problem, the report concludes that, '...It is not yet possible to state definitely a reasonable allowance for fat in the diet or to indicate the characteristics of a fatty acid mixture most favourable for the support of health. A diet selected from a wide variety of foodstuffs, both vegetable and animal, is most likely to maintain good health.' Further indications of the present status of knowledge of the various factors in the etiology of atherosclerotic heart diseases may be gained from a number of recent reviews (e.g. (7,8,9,10)).

The other topic, the contamination of milk and its products with radio-active isotopes, presents a more disquieting picture, not that there is any very real problem at the present time but rather that the increasing rate of contamination may well outstrip our knowledge of its hazards. This growing concern throughout the world is reflected in the very rapidly developing literature in this field. Nutritional hazards associated with radio-active contamination have been the subject of a recent Nutrition Society Symposium (11) in which our lack of the knowledge necessary to make any accurate estimate of the dangers both now and in the future was stressed, and a policy of more research work and less talk was advocated. Apart from investigations relating to the Windscale accident in 1957 (e.g. (12)) which illustrate the risk of a sudden severe contamination (in this case with ^{131}I) of dairy products to the extent that they were rendered unsafe for human consumption, most work on milk has centred round its ^{90}Sr content. This of course stems from the important place of milk as a source of calcium in the human diet and, as a result, the fact that the levels of ^{90}Sr in milk and its products virtually control the amounts of this isotope deposited in human bones. Literature covering the movement of ^{90}Sr from the radio-active fall-out through the soil, into the pastures, and eventually into milk is not relevant to this review, but it is clear that the level of ^{90}Sr in milk has increased over recent years and this increase, if unchecked, must eventually constitute a serious nutritional hazard (e.g. (13,14,15,16,17)). Of a number of factors which will, however, contribute some useful degree of protection, the ability of the cow to discriminate against strontium in milk production is probably the most significant. It would appear also that the removal of ^{90}Sr from contaminated milk is feasible, even on a commercial scale, using ion exchange resins (18,19). Natural causes of radio-activity in milk and the effects of nuclear fall-out have been fully reviewed by McWeeny (20).

NUTRITIVE VALUE OF MILK FOR MAN

Reports for milk production during 1957 and 1958 indicate that the post-war upward trend is still being maintained. The estimated world increase for 1957 over the previous year was 2% (21) with a further increase of 1% in 1958 (22). Associated with these increases have been slight changes in the general pattern of milk utilization. The year 1957, with an increase of 4% over the 1956 figure, set a post-war record for butter production (23) and there was a further increase of 3% in 1958 (24). Corresponding increases in cheese production were 4% in 1957 and 1% in 1958. From the many reviews of production and utilization of dairy products throughout the world, those reported in (25,26,27) supply the type of information with which the nutritionist is usually concerned.

The XVth International Dairy Congress was held in London during the period under review, but on this occasion the nutritive value of milk and its products would seem to have attracted less attention than hitherto. Nevertheless, the reports of the Congress contain much of at least indirect nutritional interest and a few strictly nutritional contributions are referred to later.

(*a*) *Adult nutrition*

Recent work on the nutritive value of milk and its products has been reviewed by several workers. Dědek & Lukáš (28) discuss the place of milk in human nutrition and make special reference to the therapeutic uses of certain special milks. They also discuss the problem of arteriosclerosis in relation to the consumption of dairy products. Milk and its uses in medicine, dietetics and nutrition have also been fully reviewed (29,30), and a number of the reviews and contributed papers presented at the Heinz International Symposium on Nutrition (31) have a direct bearing on the place of milk in both adult and infant nutrition.

de Haas (32), in a review on the nutritive value of milk protein and milk fat, points out that the value of the former has tended to be neglected, whereas that of the latter has been over-emphasized. This point of view will, of course, meet with general agreement. It is unfortunate that simple and convenient methods for estimating fat but not protein have long been available, and largely as a result of this fat has become, irrespective of the purpose for which the milk is to be used, the basis of payment and the criterion of herd improvement work. The current, but rather belated, attention to rapid methods for estimating proteins in milk is naturally of considerable nutritional interest.

An excellent review by Kon (33) covers the composition and nutritive value of milk, treated milks and milk products, the place of milk in national diets, and the use of milk and its products in child feeding schemes. Another very valuable contribution by the same author (34) discusses the influence of heat treatment and light on the composition and quality of milk. It seems relevant to repeat here a number of general points made by Kon on the place of milk in the diet and on the controversial subject of the effect of processing on the nutritive value of milk and its products; they are largely self-evident but need frequent restatement, since they tend to be lost sight of in some workers' enthusiasm for certain pet theories and ideas. In the first place, it detracts in no way from the importance of milk and of its contribution to

the balancing of man's dietaries, to remember clearly that for adults and children alike, it is no more a natural food than meats, cereals and vegetables, and that it must be considered not as a separate entity but along with the rest of the diet. The nutritive value of milk, as well as changes brought about by processing, must always be assessed in relation to the contribution of milk and its products to the mixed diet eaten. Viewed in this way many processing changes, which in themselves seem important, lose much of their significance; it is obviously senseless to worry about even marked destruction of a nutrient of which milk is a negligible source, or which is otherwise well supplied in a balanced diet. Furthermore, milk is not a stable commodity and some degree of processing is inherent in the provision of safe and palatable milk for human consumption. We must accept any slight deterioration in nutritive value which this entails and regard it merely as a part of the general change in quality which commences as soon as the milk leaves the udder. It is more important that our attention should be directed towards the much more significant losses which can occur due to mishandling at other stages in the journey from the cow to the table.

Turning to the nutritive value of individual milk products, Teply, Derse & Price (35) have shown that the treatment of milk with hydrogen peroxide followed by catalase has no effect on the composition and nutritive value of cheese produced from it. The nutritive value of sweetened condensed milk has been further investigated (36). Its protein quality was found to be not significantly different from that of fresh milk and did not decrease after 26 months' storage. The beneficial effect of supplements of dried skim-milk in cases of human undernourishment is further exemplified by two reports (37,38). The growing interest in the ultra-high-temperature treatment of milk has led to further studies on its nutritive value. Ogasa, Ishii, Kazuyori, Tanaka & Maeno (39) found it to be virtually the same as milk pasteurized at a lower temperature. Kuliev (40) has investigated the nutritive value of 'chal', a fermented camel's milk.

The Dairy Congress has once again produced a number of papers dealing with the consumption and nutritive role of milk and milk products in various parts of the world. Countries covered include Austria (41), Israel (42), French Equatorial Africa (43) and New Zealand (44). While price is generally accepted as a major factor influencing utilization, Valen (45), in a statistical survey of milk consumption in Norway, concludes that even a 25% price change gives little explanation for changes in the consumption of whole milk. Interesting reports on the success of milk sales promotion schemes in Great Britain have appeared (46,47) the latter reference stressing the importance of increasing the consumption of milk as an adult beverage if we are to increase the overall consumption of milk. The need to modify milk powders to suit the individual requirements of potential users and successful experiments in this direction in Australia are discussed by Lee & Beeby (48). From the strictly economic point of view there would, however, seem to be a limit to the extent of modification, and milk products can be adapted to new uses only with the supply and demand of the product they are replacing clearly in mind.

In contrast to the ideals of those who advocate greater consumption of milk, Bettington, Hill & Morris (49), in a controversial article, suggest that the increasing incidence of certain pathological conditions is associated with the over-consumption of milk and its products. Kept to scientific journals, articles of this type present no problems but, setting out ideas contrary to those generally accepted as scientifically

sound, they possess a high potential news value and this article was in fact widely reported and commented on in the New Zealand press. Without the background of knowledge against which the value of any scientific article may be assessed, the general public is liable to be left with the uneasy feeling that it is being misled by its more orthodox nutritionists.

(*b*) *Infant nutrition*

This is a field which has again produced a very extensive literature, and although it remains the least satisfactorily evaluated section of the subject under review, there are some indications of a clearer picture emerging. Some of the problems in the nutrition of the very young are discussed in (50,51,52) and historical aspects of infant feeding are reviewed in (53). Indian workers (54,55,56) discuss the value of various milks in infant feeding and stress the need for the manufacture of infant milk foods in India. They suggest the use of suitably modified buffalo's milk for this purpose and describe a preparation which has proved satisfactory at experimental centres.

The relative nutritive value of cow's milk preparations and of human milk would seem to have attracted less attention during the period under review than in previous years. This is no doubt due to the growing realization of the shortcomings of the various methods of comparison and to the fact that, within the limitations of these available techniques, it is now very clearly established that cow's milk, intelligently modified, is for all practical purposes in no way inferior to human milk for infant feeding. It would seem now that the real need is for those organizations giving practical advice and guidance on infant nutrition to take fuller advantage of the wealth of information which has accumulated over recent years. As would be expected, however, there is ample evidence to show the superiority of human milk over unmodified or unsuitably modified milk of other species. This should not confuse the real issue.

Surveys covering large groups of children have shown a beneficial effect of cow's milk on skeletal development, children weaned early being significantly taller and showing more ossification centres than those breast-fed for longer periods (57,58). The total base conservation is also greater in infants fed cow's milk than in those fed human milk (59). On the basis of weight gains, Jochims (60) found that sterilized milk was inferior to pasteurized milk and using the same criterion, Vignetti & Sbraccia (61) also report some superiority for a humanized cow's milk over human milk. Human milk, as compared with various cow's milk preparations exercised no protective or therapeutic effect in experiments with children suffering from gastro-enteritis (62).

In much of the infant nutrition work, just as is the case with adult nutrition, one is inclined to wonder if sufficient note is taken of the variability of the products under consideration. Milk and its products must vary considerably from time to time and from one locality to another, and the small differences in nutritive value found in some individual trials can only with extreme caution be extended to the general case. Although Walker(63) is of the opinion that the significance of dietary factors in human lactation has been over-emphasized in the past, it would seem reasonable to anticipate a much greater variability in the composition of individual human milks than in bulked cow's milks and this relatively greater uniformity in the latter may well explain many of its observed advantages.

There is a growing interest in essential fatty acids in infant nutrition (64, 65, 66, 67). It would seem that the optimum level of linoleic acid is about 4 % of the total calorie intake, but here again evaluation of the role of these acids must await a better understanding of fat metabolism in general.

The problem of milk allergy is also arousing considerable interest. A number of clinical reports have appeared (68, 69, 70, 71, 72) and the subject has been reviewed by Fries (73) who suggests that the allergy is mainly associated with the lactalbumin fraction of the milk and can frequently be overcome by denaturing these proteins by boiling. However, in other cases heat treatment of the milk is not always effective (72) so that other factors may be involved. It would seem that the incidence of true cow's milk allergy is in the order of 1 % or less (70, 71) and in these cases use of the milk of other mammals rather than preparations such as soya-bean milk is recommended (73). There is in fact one report of vitamin A deficiency in an allergic infant fed unsupplemented soya-bean milk (74).

The nature of the bifidus factor in human milk is reviewed by Petuely (75) and Gyllenberg & Carlberg (76) have investigated the growth requirements of strains of *Lactobacillus bifidus* isolated from the faeces of infants fed human or cow's milk.

There is an encouraging report by Harney (77) on the value of the dried skim-milk supplied by U.N.I.C.E.F. in reducing malnutrition in infants and children on the colony of St Kitts, Nevis Anguilla.

MILK FAT

During the period under review only a very limited amount of original work would seem to have been published on the nutritive value of milk fat. There have been, however, a number of useful reviews and texts. Thus Kummerow (78) has reviewed the nutritional characteristics of milk fat and other fats and, dealing with the problem of atherosclerosis, stresses the nebulous nature of the evidence for linking dietary fat with the increasing incidence of heart disease. The third volume of Duel's comprehensive text *The Lipids*, most regrettably a posthumous publication, has a large section on the nutritive value of fats (79). Much general information on fats, their properties, nutritive value, etc., is contained in other reviews (33, 80, 81) and tests (82, 83). Recent work on the origin of milk fat is reviewed by Glascock (84).

Milk fat has featured in a number of studies on the relationship between serum cholesterol levels and the quantities and natures of dietary fats. Keys, Anderson & Grande (85) derive equations for predicting changes in serum cholesterol relative to changes in dietary fats, but from other work such a simple and general relationship would not seem to be clearly established (86, 87, 88). The chemistry of lipids as related to atherosclerosis was the subject of a recent symposium (89). The problem is clearly one of fundamental importance and considerably more work is required to establish the influence of dietary fat on serum cholesterol levels, and indeed whether serum cholesterol levels have any simple and direct bearing on the incidence of coronary diseases. It must be remembered that the quantity of cholesterol synthesized within the body is ten to twenty times higher than the normal intake of cholesterol from a mixed diet. Milk contains other protective substances which tend to inhibit the deposition of cholesterol in arterial walls and contribute to the resistance of the arterial walls against degenerative diseases. Experiments with human volunteers on

well balanced diets have shown that an intake of 50 g milk fat from milk, cream and cheese led to significant decreases of cholesterol in the blood (2). The same beneficial effects might not be obtained from typical margarine fats with their higher molecular weights and increased content of saturated fatty acids (3). On the other hand, however, in experiments with pigs (90), the incidence of atherosclerosis was higher in those animals fed milk fat than in those receiving a corresponding amount of margarine. It would seem significant that neither milk fat nor margarine raised the blood cholesterol or phospholipid levels above those found in control animals fed a low-fat diet. A recent text (91) contains much valuable information on cholesterol and deals with blood levels and their relation to diseased states.

FAT SOLUBLE VITAMINS

(*a*) *Vitamin A and carotene*

In general the methods for the estimation of carotene and vitamin A in dairy products seem to have become well established and few new methods or modifications are being reported. A method involving direct saponification of the milk product without preliminary fat extraction is described by Wilkinson & Conochie (92). For particular products methods of this type are simple and appear to give relatively good recoveries of added vitamin A.

Levels of vitamin A and carotene have been reported for milk and milk fat produced in a number of areas; these include parts of the U.S.S.R. (93, 94, 95), Czechoslovakia (96), Denmark (97) and Great Britain (98). The usual seasonal variation between periods of stall and pasture feeding, in both the carotene and vitamin A content of the milk, is noted (95, 96, 98), reference (95) also recording differences in the utilization of carotene from different pastures. Thompson (98) reports the beneficial effect of kale and grass silage in raising winter vitamin A and carotene levels. The effect of breed is discussed (94, 98) and the fact that genetic factors are involved in determining the levels of carotene and vitamin A in the milk fat of individual cows is clearly established by studies on the uniformity of monozygous twins by McDowell & Patchell (99). Figures for the vitamin A content of buffalo's milk in the U.S.S.R. (100) and Rumania (101), and for ewe's milk in Japan (102) have been published.

In general these findings agree with earlier reports and in so far as cow's milk is concerned further support the observation in an earlier review (103) that the vitamin A potency of milk and milk products in various parts of the world is a reflexion of feeding and management in these areas, modified by the characteristics of the dominant breed.

Further investigating the unusual seasonal variations in the vitamin A potency of the milk fat of cows fed throughout the year on pasture of uniformly high carotene content, McGillivray, Thompson & Worker (104) have studied various factors which might influence the absorption of carotene from pasture. They found it to be influenced by the concentration of carotene in the oil used as vehicle, the degree of unsaturation of the oil, and the presence of non-digestible wax. All of these factors might be expected to vary in pastures throughout the year. The availability of carotene from different sources and the resultant effect on the vitamin A potency of the milk fat have been reported (105).

In studies on the composition of mastitis milk, Italian workers (106) found no differences between the carotene and vitamin A contents of milk from healthy and infected quarters. It should be noted, however, that earlier reports (e.g. (107)) have shown that the effect of mastitis on the vitamin content of milk depends on the severity of infection.

In connexion with the increasing use of heavy liquid paraffin for bloat control in New Zealand, McGillivray, McDowall & Reid (108) have further investigated its effect particularly on the fat-soluble vitamin content of the milk. They conclude, with some reservations, that the decrease in nutritive value of the milk is outweighed by the economic advantages of the oil in bloat control. The above work also provided an opportunity for studies on the origin of various milk constituents, particularly vitamin A and the carotenoids (109). It is suggested that while most of the carotene and vitamin A in milk is of immediate dietary origin, there is also some contribution from body reserves through the uptake by the mammary gland of the specific proteins with which carotene and vitamin A are associated in the blood. This suggestion would seem to explain observed facts regarding the fat-soluble vitamins in normal and abnormal mammary secretions.

Several reports have appeared on the stability of vitamin A and carotene during the processing and storage of dairy products. Khotsko (93) reports surprisingly high losses of both vitamin A and carotene during the storage of salted ripened-cream butter as compared with unsalted sweet-cream butter. He also notes the increased losses of vitamin A and carotene associated with the development of storage defects such as tallowy, oily, fishy and rancid flavours. Comparable results are reported by Poryadkova (110) who also found that the keeping quality of butter of high vitamin A content was better than that of butter low in vitamin A. During storage, under comparable conditions, vitamin A has been found to be more stable in buffalo's milk fat than in cow's milk fat in which it is, in turn, more stable than in colostral fat (111).

The loss of vitamins including vitamin A due to exposure of homogenized milk to light has been investigated by Birdsall, Teply & Derse (112). Foil laminated waxed paper cartons were most effective in protecting the vitamin A from destruction. Changes in the vitamin A content of 'pickled' cheeses during manufacture and ripening have been reported (113). Sokolov (114) records vitamin A losses of about 17 % during the processing of a concentrated sterilized milk. There was a further loss of about 10 % during storage at room temperatures for 6 months.

Synthetic β-carotene is finding increasing use in the colouring of dairy products. The low solubility of carotene in oils (about 0·1 %) presents problems since the addition of the relatively large quantities of the oil used as a vehicle for the carotene may cause flavour defects in dairy products (115). However, micro-pulverized preparations containing up to 30 % of β-carotene are now available for use in cheese-making and continuous butter-making and there is also a thixotropic gel containing up to 10 % β-carotene for use in batch butter-making (116). Water dispersible forms are also available for cheese and ice-cream manufacture (117). While the quantities added are not great, the synthetic β-carotene does contribute to the nutritive value of the product particularly as in many cases the natural vitamin A potency before colouring has been relatively low.

(*b*) *Vitamin D*

A chemical method is described for the estimation of vitamin D in evaporated milk (118). It depends on the specific inhibition of the vitamin D—antimony trichloride reaction with acetic anhydride. Many workers will welcome the introduction of chemical tests of this type should they prove applicable to the very low concentrations of vitamin D normally encountered in dairy products.

Marked seasonal variations have been reported in the vitamin D content of milk (119). Levels ranged from 3·3 to 23·5 i.u. vitamin D/kg milk and there was a marked rise when cows went from stall-feeding on to pasture. It is considered, however, that the effect of changes in feeding is slight compared with that of exposure to sunlight.

(*c*) *Vitamin E*

Handwerk & Bird (120) and Worker (121) report methods for the chemical estimation of tocopherols in dairy products. Both methods involve saponification with pyrogallol followed by chromatography. Worker's method is an extension of a standard method for the estimation of carotenoids and vitamin A and these substances may also be estimated, along with the tocopherol, in the one assay.

The tocopherol content of the milk from different breeds of cows was found by Russian workers to range from 87 to 103 μg/100 ml milk (94). Ward (122) has compared the tocopherol content of samples of margarines and butters. The butters contained 14–20 μg tocopherol/g and the margarines from 7 to 158 μg/g depending on the fats used in their manufacture.

The relatively high losses of fat-soluble vitamins, and of course the associated deterioration of the fat itself, from ghee during manufacture and storage under the conditions obtaining at the present time in many parts of India is still a pressing problem. The addition of the anti-oxidants, propyl gallate and butylhydroxyanisole, has no effect on the rate of tocopherol loss during storage or heating (123). The same workers (124) have also suggested that the tocopherol content of ghee might be used as an index of adulteration. Normal ghee was found to contain between about 20 and 45 μg tocopherol/g and the method enables adulteration above about 10 % to be detected.

(*d*) *Vitamin fortification*

The fortification of foods, particularly dairy products, with vitamins is still a controversial topic. Argument continues over the merits of fortification, the products to be fortified, the amount and nature of the vitamin preparations to be used, and the methods of fortification. The whole subject is well reviewed by Harris (125) and the pros and cons of vitamin D fortification are discussed by Müller (126). Vitamins are added to human foods either with the aim of restoring to natural levels the vitamins lost during processing or storage or with the idea of fortification of the food, usually considerably above natural levels, to make good a dietary deficiency. There seems little ground for accepting the former but opposing the latter. The natural vitamin content of a food is designed for a biological need which bears no relation to human infant or adult nutrition and if milk and its products are to be fortified it seems logical to fortify to an extent that will, bearing in mind the place of milk in human dietaries,

help materially to satisfy daily requirements. However, unless carefully controlled, there is the risk of over fortification particularly of infant foods and at the present time this is thought to be a factor in the incidence of hypercalcaemia (127). Case histories are quoted showing intakes by some infants of over 2000 i.u. vitamin D/day from fortified foods (128). Considering all the factors involved, Holtz & Hiller (129), in a discussion of human vitamin D requirements, recommend the fortification of milk with vitamin D and consider that the preferred method is still irradiation with ultra-violet light. Such irradiation has no effect on the natural vitamin A content of the milk (130).

Interest continues in the stability of added vitamins during the processing and storage of fortified dairy products. Conochie & Wilkinson (131) report good stability of both the natural and added vitamin A in fortified sweetened condensed milk for up to 6 months irrespective of storage temperature. The same workers (132) found that considerable losses of vitamin A occurred during the heat treatment of reconstituted fortified skim-milk powders. Light also caused marked losses of vitamin A from similar reconstituted powders, but the effect was reduced when hydrogenated coconut oil was homogenized into the reconstituted milk. Losses of vitamin A from these 'filled' milks were no greater than from normal whole milk similarly exposed (133). Other findings on the stability of vitamin A in reconstituted, fortified skim-milk powder are given in (134). The stability of solutions of vitamins A and D suitable for use in the fortification of milk and its products has been reported (135).

REFERENCES

(1) McGillivray, W. A. & Porter, J. W. G. (1958). *J. Dairy Res.* **25**, 344.
(2) Halden, W. (1959). *Proc. XV Int. Dairy Congr.* **1**, 27.
(3) Italicus, (1959). *Latte Aliment.* **6**, 3.
(4) Bloem, T. F. (1958). *Ned. melk-en Zuiveltydschr.* **12**, 278.
(5) den Hartog, C. (1958). *Ned. melk-en Zuiveltydschr.* **12**, 360.
(6) Recommended Dietary Allowances, Rev. (1958). *Publ.* 589, *National Academy of Sciences—National Research Council, Washington D.C.*
(7) James, A. T. & Lovelock, J. E. (1958). *Brit. med. Bull.* **14**, 262.
(8) Groen, J. (1958). *Ned. melk-en Zuiveltydschr.* **12**, 282.
(9) Davis, J. G. (1959). *Lancet*, **1**, 631.
(10) Anderson, Z. E. (1959). *J. Dairy Sci.* **42**, 1401.
(11) *Proc. Nutr. Soc.* (1959). **18**, 34.
(12) Cmd. 302. (1957). H.M. Stationery Office.
(13) Merten, D. & Knoop, E. (1958). *Kieler Milchwirtschaft. FortschBerichte*, **10**, 1.
(14) Bryant, F. J., Chamberlain, A. C., Spicer, G. S. & Webb, M. S. W. (1958). *Brit. Med. J.* **1**, 1371.
(15) Bryant, R. J., Chamberlain, A. C., Morgan, A. & Spicer, G. S. (1956). *A.E.R.E.* HP/R 2056.
(16) Russell, R. S. (1958). *Nature, Lond.*, **182**, 834.
(17) Eckelmann, W. R., Kulp, J. L. & Schulert, A. R. (1958). *Science*, **127**, 266.
(18) Demott, B. J. & Cragle, R. G. (1959). *J. Dairy Sci.* **42**, 897.
(19) *Amer. Milk Rev. & Milk Pl. Mon.* (1959). **21**, 84.
(20) McWeeny, D. J. (1959). *Dairy Engng*, **76**, 95, 110.
(21) *Foreign Agric. Circ. U.S. Dep. Agric.* (1958) FD 14–58.
(22) *Foreign Agric. Circ. U.S. Dep. Agric.* (1959) FD 10–59.
(23) *Foreign Agric. Circ. U.S. Dep. Agric.* (1958). FD 7–58.
(24) *Foreign Agric. Circ. U.S. Dep. Agric.* (1959). FD 8–59.
(25) *Production Yearbook* (1958). Volume 12: Food and Agriculture Organisation of the United Nations.
(26) *Dairy Produce* (1957). A review of production, trade, consumption and price relating to butter, cheese, condensed milk, milk powder, casein, eggs, egg products and margarine. Commonwealth Economic Committee, Intelligence Branch. H.M. Stationery Office.
(27) *Dairy Produce* (1958). A review of production, trade, consumption and price relating to butter, cheese, condensed milk, milk powder, casein, eggs, egg products and margarine. Commonwealth Economic Committee, Intelligence Branch. H.M. Stationery Office.

(28) Dědek, M. & Lukáš, A. (1959). *Průmysl. Potravin*, **10**, 266.
(29) Goded y Mur, A. (1957). *An. Inst. Hig. Zaragoza*, **6** (2), 1.
(30) Davis, J. G. (1958). *Dairy Ind.* **23**, 468, 496.
(31) Proceedings of the Heinz International Symposium on Nutrition (1959). *Fed. Proc.* **18** (2), Pt. II, 1.
(32) de Haas, J. H. (1958). *Voeding*, **19**, 343.
(33) Kon, S. K. (1959). *F.A.O. nutr. stud.* no. 17.
(34) Kon, S. K. (1958). *Dairy Sci. Abstr.* **20**, 887.
(35) Teply, L. J., Derse, P. H. & Price, W. V. (1958). *J. Dairy Sci.* **41**, 593.
(36) Guezennec, J., Girard, P. & Lanteaume M. Th. (1958). *Lait*, **38**, 369.
(37) Spies, T. D. (1958). *Amer. J. med. Ass.* **167**, 675.
(38) Spies, T. D., Dreizen, S., Snodgrasse, R. M., Arnett, C. M. & Webb-Peploe, H. (1959). *J. Dis. Child.* **98**, 187.
(39) Ogasa, K., Ishii, K., Kazuyori, H., Tanaka, S. & Maeno, M. (1959). *Proc. XV Int. Dairy Congr.* **1**, 405.
(40) Kuliev, K. A. (1958). *Vop. Pitan.* **17**, 90.
(41) Erhart, G. (1959). *Proc. XV Int. Dairy Congr.* **4**, 2313.
(42) Kleinfield, M. R. (1959). *Proc. XV Int. Dairy Congr.* **4**, 2325.
(43) Veisseyre, R. (1959). *Proc. XV Int. Dairy Congr.* **4**, 2350.
(44) White, J. V. (1959). *Proc. XV Int. Dairy Congr.* **4**, 2356.
(45) Valen, A. L. (1959). *Proc. XV Int. Dairy Congr.* **4**, 2334.
(46) Fortescue, T. V. N. (1959). *Proc. XV Int. Dairy Congr.* **4**, 2367.
(47) Whitehead, B. T. (1959). *Proc. XV Int. Dairy Congr.* **4**, 2372.
(48) Lee, J. W. & Beeby, R. (1959). *Proc. XV Int. Dairy Congr.* **2**, 1143.
(49) Bettington, R. H., Hill, B. H. R. & Morris, E. A. (1957). *N.Z. med. J.* **56**, 577.
(50) Smith, C. A. (1958). *Proc. Nutr. Soc.* **17**, 50.
(51) Hytten, F. E. (1958). *Proc. Nutr. Soc.* **17**, 57.
(52) Jonxis, J. H. P. (1958). *Voeding*, **19**, 547.
(53) Schadewoldt, H. (1957). *Dtsch. med. Wschr.* **82**, 1621.
(54) Chandrasekhara, M. R., Bhagawan, R. K., Swaminathan, M. & Subrahmanyan, V. (1957). *Indian J. Child Hlth*, **6**, 701.
(55) Chandrasekhara, M. R., Naragana Rao, M., Swaminathan, M., Bhatia, D. S. & Subrahmanyan, V. (1959). *Proc. XV Int. Dairy Congr.* **2**, 1145.
(56) Subrahmanyan, V., Chandrasekhara, M. R. & Swaminathan, M. (1958). *Indian J. Pediat.* **25**, 173.
(57) Mellander, O. & Vahlquist, B. (1957). *Amer. J. Clin. Nutr.* **5**, 493.
(58) Vahlquist, B., Mellander, O. & Wickland, H. (1957). *Ann. Paediat. Fenn.* **3**, 513.
(59) Fomon, S. J., Harris, D. M. & Jensen, R. L. (1958). *J. Dis. Child.* **96**, 592.
(60) Jochims, J. Z. (1957). *Kinderheilk.* **80**, 1.
(61) Vignetti, P. & Sbraccia, C. (1958). *Lattante*, **29**, 172.
(62) Lambrechts, A., Hainaut, H. & Nicholas-Goldstein, M. (1957). *Pr. méd.* **65**, 1607.
(63) Walker, A. R. E. (1958). *Ann. N.Y. Acad. Sci.* **69**, 989.
(64) Wiese, H. F., Hansen, A. E. & Adam, D. J. D. (1958). *J. Nutr.* **66**, 345.
(65) Adam, D. J. D., Hansen, A. E. & Wiese, H. F. (1959). *J. Nutr.* **66**, 555.
(66) Hansen, A. E., Haggard, M. E., Boelsche, A. N., Adam, D. J. D. & Wiese, H. F. (1959). *J. Nutr.* **66**, 565.
(67) Hansen, A. E. (1958). *J. Amer. diet. Ass.* **34**, 239.
(68) O'Beirne, R. M. (1958). *Brit. med. J.* **2**, 635.
(69) Bower, B. D., Stanley-Roose, D. G. & Wolff, O. H. (1958). *Brit. med. J.* **2**, 195.
(70) Munda, F. del, Rodriguez, S. & Oliveros, N. (1956). *J. Phil. Is. med. Ass.* **32**, 457.
(71) Bachman, K. D. & Dees, S. C. (1957). *Pediatrics, Springfield*, **20**, 393, 400.
(72) Woodcock, R. H. (1958). *Lancet*, **1**, 747.
(73) Fries, J. H. (1957). *J. Amer. med. Ass.* **165**, 1542.
(74) Wolf, I. J. (1958). *J. Amer. med. Ass.* **166**, 1859.
(75) Petuely, F. (1957). *Dtsch. med. Wschr.* **82**, 1957.
(76) Gyllenberg, H. & Carlberg, G. (1958). *Acta. path. microbiol. Scand.* **42**, 380.
(77) Harney, L. (1958). *W. Ind. med. J.* **7**, 211.
(78) Kummerow, F. A. (1957). *J. Dairy Sci.* **40**, 1350.
(79) Deuel, H. J. Jr. (1957). *The Lipids*, Vol. III. New York and London: Interscience Publishers Inc.
(80) Kinsell, L. W. (1958). *Amer. J. clin. Nutr.* **6**, 583.
(81) Piskur, M. M. (1958). *J. Amer. Oil Chem. Soc.* **35**, 288.
(82) Holman, R. T., Lundberg, W. O. & Malkin, T. (editors) (1957). *Progress in the Chemistry of Fats and Other Lipids*. Vol. IV. London, New York, Paris: Pergamon Press.

(83) Sinclair, H. M. (editor) (1958). Essential fatty acids. *Proceedings of the Fourth Internationa Conference on Biochemical Problems of Lipids*, July 1957. London: Butterworths Scientific Publications.
(84) Glascock, R. F. (1958). *Proc. roy. Soc.* B, **149**, 402.
(85) Keys, A., Anderson, J. T. & Grande, F. (1957). *Lancet*, **2**, 959.
(86) Gopalan, C. & Ramanathan, K. S. (1958). *Indian med. J.* **46**, 473.
(87) Beveridge, J. M. R., Connell, W. F., Haust, H. L. & White, M. (1958). *Int. Congr. Biochem. (Suppl. Int. Abstr. biol. Sci.)* p. 204.
(88) Padmavati, S., Gupta, S. & Pantulu, G. V. A. (1958). *Indian J. med. Res.* **46**, 245.
(89) Page, I. H. (editor) (1958). *Chemistry of Lipids as Related to Atherosclerosis. A Symposium.* Springfield, Illinois: Charles C. Thomas.
(90) Rowsell, H. C., Downie, H. G. & Mustard, J. F. (1958). *Canad. med. Ass. J.* **79**, 647.
(91) Kritchevsky, D. (1958). *Cholesterol.* New York: John Wiley and Sons.
(92) Wilkinson, R. A. & Conochie, J. (1958). *Aust. J. Dairy Tech.* **13**, 32.
(93) Khotsko, Y. A. (1958). *Dokl. vses. Konf. Moloch. Delu*, p. 296.
(94) Ermakova, M. A. (1958). *Dokl. vses. Konf. Moloch. Delu*, p. 109.
(95) Ermakova, M. A. (1958). *Dokl. vses. Konf. Moloch. Delu*, p. 177.
(96) Gažo, M. & Landau, L. (1958). *Sborn. čsl. Akad. Zeměd. Věd.* **31**, 679.
(97) Bro-Rasmussen, F. (1956). *Statens Hushold-ningsvåds Fazlige Medd.* no. 10, p. 37.
(98) Thompson, S. Y. (1959). *Proc. XV Int. Dairy Congr.* **1**, 247.
(99) McDowall, F. H. & Patchell, M. R. (1958). *J. Dairy Res.* **25**, 159.
(100) Akhundov, D. M. (1958). *Dokl. vses. Konf. Moloch. Delu*, p. 247.
(101) Vermesanu, N., Mogos, E. & Rosca, N. (1958). *An. Inst. Cercet. Zootehn.* **15**, 263.
(102) Nakaniski, T. & Tokita, F. (1957). *J. agric. Res.* **8**, 155.
(103) McGillivray, W. A. & Thompson, S. Y. (1957). *Proc. Nutr. Soc.* **16**, 30.
(104) McGillivray, W. A., Thompson, S. Y. & Worker, N. A. (1958). *J. Dairy Res.* **25**, 439.
(105) Bullis, D. D., Weswig, P. H., Jones, I. R. & Bone, J. F. (1958). *Proc. W. Div. Amer. Dairy Sci. Ass.* 1958.
(106) Marmori, M. G., Nani, S. & Redaelli, G. (1957). *Atti Soc. ital. Sci. vet.* **11**, 804.
(107) McGillivray, W. A. (1957). *J. Dairy Res.* **24**, 352.
(108) McGillivray, W. A. McDowall, F. H. & Reid, C. S. W. (1959). *N.Z. J. agric. Res.* **2**, 35.
(109) McGillivray, W. A. (1959). *N.Z. J. agric. Res.* **2**, 694.
(110) Poryadkova, K. (1957). *Mol. Prom.* **18** (10), 42.
(111) Narayanan, K. M. & Anantakrishnan, C. P. (1958). *Indian J. Dairy Sci.* **10**, 165.
(112) Birdsall, J. J., Teply, L. J. & Derse, P. H. (1958). *Food Tech., Champaign*, **12**, 670.
(113) Sabry, Z. I. & Guerrant, N. B. (1958). *J. Dairy Sci.* **41**, 925.
(114) Sokolov, F. (1957). *Mol. Prom.* **18** (12), 20.
(115) Stüssi, D. (1957). *Schweiz. Milchztg*, **83**, 430, 433.
(116) Bauernfeind, J. C., Smith, E. G. & Bunnell, R. H. (1958). *Food Tech., Champaign*, **12**, 527.
(117) Bunnell, R. H., Driscoll, W. & Bauernfeind, J. C. (1958). *Food Tech., Champaign*, **12**, 536.
(118) Jones, S. W., Morris, W. W. & Wilkie, J. B. (1959). *J. Ass. off. Agric. Chem., Wash.*, **42**, 180.
(119) Lenkeit, W., Brune, H. & Günther, K. (1958). *Z. Tierphysiol. Tierernakrung. Futtermillelk.* **13**, 247.
(120) Handwerk, R. L. & Bird, E. W. (1958). *J. Dairy Sci.* **41**, 28.
(121) Worker, N. A. (1958). *J. Sci. Fd Agric.* **9**, 122.
(122) Ward, R. J. (1958). *Brit. J. Nutr.* **12**, 231.
(123) Ramanujam, R. A. & Anantakrishnan, C. P. (1958) *Indian J. Dairy Sci.* **11**, 179.
(124) Ramanujam, R. A. & Anantakrishnan, C. P. (1958). *Curr. Sci.* **27**, 255.
(125) Harris, R. S. (1959). *J. Agric. Fd Chem.* **7**, 88.
(126) Müller, E. (1957). *Dtsch. med. Wschr.* **82**, 152, 179.
(127) MacDonald, A. M. (1958). *Proc. Nutr. Soc.* **17**, 74.
(128) Mitchell, R. G. (1958). *Proc. Nutr. Soc.* **17**, 71.
(129) Holtz, F. & Hiller, F. K. (1958). *Fette, Seifen, Anstrichmitt*, **60**, 280.
(130) Rogick, F. A. (1957). *Bol. Indústr. Amin.* **16**, 185.
(131) Conochie, J. & Wilkinson, R. A. (1958). *Aust. J. Dairy Tech.* **13**, 27.
(132) Wilkinson, R. A. & Conochie, J. (1958). *Aust. J. Dairy Tech.* **13**, 29.
(133) Conochie, J. & Wilkinson, R. A. (1958). *Aust. J. Dairy Tech.* **13**, 74.
(134) Anantakrishnan, C. P. & Conochie, J. (1958). *Aust. J. Dairy Tech.* **13**, 151.
(135) Souci, S. W. & Schillinger, A. (1958). *Milchwissenschaft*, **13**, 162.

ALPHABETICAL LIST OF REFERENCES

Adam, 64, 65, 66; Akhundov, 100; *Amer. Milk Rev.* 19; Anantakrishnan, 111, 123, 124, 134; Anderson, 10, 85; Arnett, 38.

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PART 2

INTRODUCTION

This section of the review is based on a selection of papers published in the main during 1958 and 1959, and concerned with the proteins, minerals and water-soluble vitamins of milk and milk products.

MILK PROTEINS

General

Average, maximum and minimum values for the amino-acid content of cow's, goat's, human and buffalo's milk and for casein, various cheeses, whey and lactalbumin are included in a new set of tables of the amino-acid content of foods (1).

Biological value

General problems concerned with the nutritive value of proteins were discussed at a Nutrition Society Symposium in 1957: Henry & Kon (2) reviewed the significance of amino-acid composition, the mutual supplementary effects of proteins and the

effect of heat and storage on the biological value of proteins; Bender (3) considered the various biological methods available for determining the nutritive value of proteins, and pointed out that all the methods give a measure of quality that is a function of the limiting amino acid and do not yield any information about the other amino acids and thus about the potential value of the protein in combination with other proteins; Carpenter (4) outlined some of the physical and chemical tests that have been used to estimate protein quality and discussed the application to the grading of proteins of his dinitrofluorobenzene test for available lysine, and Platt & Miller (5) discussed general problems of the quantity and quality of proteins in human nutrition.

The biological value of proteins for rats is usually determined with an arbitrary concentration, usually 8–10 %, of protein in the diet. However, the biological value of a protein may alter with changes in its concentration in the diet. Thus, Forbes, Vaughan & Yoke (6) determined the biological value for growing rats of vitamin-free casein at ten levels of protein intake and found that it declined steadily from 92 at a 4·2 % level of intake to 47 when casein comprised 28·9 % of the diet. Platt & Miller (5) quote similar findings with casein which show a decline from 60 at an 8 %, to 38 at a 28 % level of intake.

Further findings of the effect of different methods of preservation or processing on the biological value of milk proteins are reported. Henry & Porter (7) found that the biological values for growing rats of the proteins of raw skim-milk, skim-milk treated with 1 % formalin, rennet whey with lactose and minerals partly removed, and this whey after UHT sterilization were 88, 87, 99 and 99, respectively. The four products were freeze-dried after preparation and fed to supply an 8 % level of protein in the diet. Causeret, Hugot & Mocquot (8) determined the biological value for rats of skim-milk, calcium phosphocaseinate, calcium paracaseinate and acid casein as 78, 67, 65 and 66, which confounded an earlier report (9) that the clotting of milk with rennin reduced the biological value of the protein in the clot. Growth tests with young rats and balance studies with adult rats showed that the protein efficiency ratio, biological value and digestibility of the proteins of sweetened condensed milk were not significantly different from those of fresh milk, and did not decrease significantly after 26 months' storage although the colour and consistency of the milk changed considerably (10). Ney (11) compared the growth and reproductive performance through three generations of rats given evaporated milk sterilized in-can at 247 °F for 15 min and supplemented with minerals and vitamin D, evaporated milk pasteurized by HTST and similarly supplemented, or a diet of dried skim-milk and vegetable fat supplemented with vitamins and minerals and HTST pasteurized. The performance of the rats was best on the dried skim-milk diet and poorest on the in-can sterilized evaporated milk. Although he measured the vitamin content of the diets and considered them adequate and not a factor responsible for the results, it is noteworthy that the poorest performance was on the diet with the lowest content of vitamin B_{12}. Thus, in view of the known destruction of vitamin B_{12} during in-can sterilization of evaporated milk (12), it seems very probable that the findings may merely reflect the inadequacy of the vitamin B_{12} content of the diets.

One of the difficulties attending the assessment with rats of the nutritive value of the proteins of human milk, and of diets that resemble it in composition, is the high

ratio of lactose to protein that such diets contain. This high level of lactose frequently causes diarrhoea in the rats and thus reduces the accuracy of the results of nitrogen balance or growth tests. Tomarelli, Minnick, d'Amato & Bernhart (13) have made a careful comparison of the nutritive value for rats of the proteins of cow's and human milk after reducing the lactose content of the latter either by crystallization or by dialysis. Rat growth tests, at a 10 % level of protein intake with isonitrogenous, isocaloric diets containing the same amount of lactose, and nitrogen balance studies at an 8 % level of protein, showed no significant difference between the proteins of the two milks in protein efficiency ratio, protein retention efficiency, net protein utilization, biological value and true digestibility. A different approach was followed by Diaz, Speer, Homeyer, Hays & Catron (14) who investigated the possibility of using as the test animal the young pig which, unlike the rat, can efficiently utilize diets containing lactose as the main source of energy. They compared the gain in weight and food efficiency of 7 to 10-day-old pigs receiving commercially available milk formulae for infants with those of pigs given normal prestarter rations. Although the performance of their animals was somewhat erratic, their results show that satisfactory growth was obtained when the formulae contained milk proteins and were fed at levels to supply 2·7–3·4 % of protein in the liquid diet, but lower levels of milk proteins or formulae containing soya protein were less satisfactory. An indirect method of assessing the nutritive value of such products was explored by Dju, Baur & Filer (15) who determined the xanthine oxidase activity in the livers of rats receiving different dietary proteins, and showed that this method could be successfully used to rank the nutritive value of egg protein, an infant food containing whole milk proteins, casein, gluten and zein in that order.

The deleterious effect of radiation sterilization on milk proteins has been further studied by Tsien & Johnson (16) who determined the amino-acid composition of acid hydrolysates of the proteins of evaporated milk before and after irradiation at dose levels of 2·8 or 9·3 Mrad. The lower level of irradiation caused significant losses of aspartic acid, serine, methionine and isoleucine and the higher level caused further significant losses of aspartic acid, glutamic acid, glycine, methionine, isoleucine, phenylalanine and histidine. These findings do not demonstrate the loss of cystine reported previously on the basis of rat tests (17), though it is possible that irradiation causes conversion of cystine to a nutritionally unavailable form which is measured as cysteic acid and hence calculated as cystine in the amino-acid determination.

The sequence in which the amino acids of casein become limiting for the growth of young rats fed on diets containing 6 % of casein supplemented with various amino-acid mixtures has been worked out by Harper (18). The most limiting acids are methionine and cystine, followed by threonine, then tryptophan, isoleucine, leucine, histidine, valine and phenylalanine all about equally limiting, with lysine slightly less so, and finally arginine. He points out that as this order differs from that calculated from the known amino-acid composition of casein and the accepted amino-acid requirements of the rat, it implies either that insufficient is known of these requirements or that the amino acids of casein are not all fully available.

Availability of amino acids from milk proteins

Milk proteins are generally well digested. In fact Gupta, Dakroury & Harper (19) showed that casein was digested and assimilated by rats as rapidly as an amino-acid mixture. Nitrogen balance studies with young men indicated, except for a slightly higher value for isoleucine from egg, a generally similar availability of essential amino acids from cottage cheese, egg, and egg and milk; the apparent lower availability from raw milk was almost certainly due to its tendency to cause diarrhoea in some of the subjects (20).

It now seems well established that the unavailability of lysine after the Maillard reaction in heat-treated milks is due to difficulty in the enzymic liberation of the amino acid. Two methods have been used to study the availability of lysine for rats. Gupta, Dakroury, Harper & Elvehjem (21) examined its availability from dried milks by determining the value of the milks as supplements to a wheat gluten diet for growing rats. The percentage availability of lysine from the protein was estimated by comparing the growth responses (weight gained per 100 g of food consumed) obtained with the protein supplement and with graded levels of crystalline lysine. They found that whereas lysine was 100 and 92 % available from casein and spray-dried skim-milk, it was only 68 % available from roller-dried skim-milk. Mauron & Mottu (22) obtained similar results from rat growth tests in which milk proteins supplemented with methionine were the sole protein component of the diet. With a 10 % level of protein in the diet they were able to show that the relationship between *in vitro* lysine availability (23) and decrease in protein efficiency is essentially linear. Thus a series of roller-dried skim-milks showed by the *in vitro* procedure deteriorations of from 18 to 72 % and decreases in protein efficiency of from 3 to 82 %. The protein efficiency could be almost completely restored by an adequate supplement of lysine. They emphasize the important point that the addition of methionine increases the sensitivity of the rat assay to lysine deterioration, for when this deterioration is small the difference between spray- and roller-dried skim-milk cannot be detected unless methionine is added to the diet. Failure to make this addition and to reduce the lactose contents of the diets may have been responsible for the lack of response to a lysine supplement shown by rats receiving an infant formula or diets based on evaporated milk in another study (24).

Supplementary relationships

Several groups of workers have further illustrated the value of milk proteins as supplements to bread proteins. Hutchinson, Moran & Pace (25) have made a careful study of the nutritive value for the rat of bread protein as influenced by the level of protein intake, the level of supplementation with L-lysine and L-threonine and the addition of egg and milk proteins. The growth rate of rats and the protein efficiency ratio with a diet containing 13 % of bread protein supplemented with 0·5 % of L-lysine and 0·2 % L-threonine was similar to that observed when the bread protein was supplemented with casein, dried skim-milk or whole egg protein. Howard, Monson, Bauer & Block (26) and Block & Mandl (27), however, consider the supplementation of wheat protein purely from the standpoint of increasing the dietary supply of lysine. Assuming that the rat requires 5·3 g of lysine per 100 g of protein

they derive a formula from which the supplemental value of a food protein and the level of 'complete protein' in the diet may be calculated. They suggest that complete protein is a more satisfactory index of nutritional value than crude or total protein. Rat growth tests (26) gave results showing a good correlation between dietary level of complete protein, growth rate and protein content of the carcass, and supplementing wheat flour with lactalbumin, dried skim-milk, soya-bean protein, wheat gluten or lysine gave results in accordance with the calculated content of complete protein.

The mutual supplementary effect of breakfast cereals and milk in rat diets has been shown (28); a supplement of 0·5 % of DL-lysine did not improve the growth of rats receiving a diet of wheat flakes, sugar and milk, though it did improve growth when the milk was not given (29).

In a nitrogen balance study with a young man, Kofrańyi (30) found that substitution of cheese for one-fifth of the protein of a purely vegetable protein diet resulted in a 39 % increase in the biological value of the dietary protein.

WATER-SOLUBLE VITAMINS

Analytical methods and compositional data

No important changes in assay procedures have been proposed during the last 2 years.

Some interesting findings were reported by Singh & Merilan (31) who measured the riboflavin, pantothenic acid, nicotinic acid and ascorbic acid contents of the blood and milk of two Friesian and two Jersey cows subjected to diurnal temperature rhythms that imitated those of normal seasonal variations. The range of temperatures used were 40–70, 60–110 and 70–100 °F. The milk of all the animals exposed to the higher temperatures contained slightly lower levels of pantothenic acid, but higher concentrations of ascorbic acid, and that of the Friesians, but not that of the Jerseys, contained higher levels of riboflavin and nicotinic acid.

Gregory, Ford & Kon (32) have published fuller details of their study of the effect of stage of lactation on the concentrations of biotin, nicotinic acid, pantothenic acid, riboflavin and vitamins B_6 and B_{12} in the milk of Shorthorn and Friesian cows.

Some other recent publications listing determinations of vitamins in milk and milk products are summarized in the following paragraphs.

No loss of thiamine, riboflavin or nicotinic acid was found in dried milk exposed to 2·79 or 5·58 Mrad of gamma radiation (33). Boiling of pasteurized milk caused slight variable losses of riboflavin (34). A normal range of values (1·4–2·7 μg/g) was found for riboflavin in six samples of milk taken in Madrid (35). The riboflavin content of twenty-four samples of raw milk in Japan averaged 2 μg/ml and of forty samples of pasteurized milk 1·8 μg/ml; normal values were also obtained for samples of dried milks (36). The pantothenic acid content of the milk of individual cows in Italy varied with the season, but not with the stage of lactation; values ranged from a maximum of 5·8 μg/ml in May to a minimum of 3·5 μg/ml in April (37). Increased levels of vitamins B_6 and B_{12} and of nicotinic acid were found in the milk from cows with mastitis (38). Davidov & Kruglova (39) made a detailed study that showed seasonal effects and inter-breed differences in the biotin content of milk from various breeds in Russia. The ranges of vitamin B_6 content of cream, milk and butter in Denmark were

29–54, 36–62 and 4 μg/100 g, respectively (40). The average level of vitamin B_6 throughout lactation of cow's milk was 76 μg/100 ml and of human milk 10 μg/100 ml (41). Exposure to sunlight for 1 or 2 h of milk in clear glass bottles caused losses of vitamin B_6 of 26–31 and 30–42 % (42). Raw milk in Switzerland contained 5·2 μg/l. of cyanocobalamin by assay with *Ochromonas malhamensis* and 6·3 μg/l. as determined by rat liver storage assay; values of 3·5, 3·3 and 2·2 μg/100 g were obtained for dried milk, acidified dried milk and acidified dried milk with carbohydrate (43).

The ascorbic-acid contents of the milk of Friesian cows in Russia ranged from 13 to 22 mg/l. (44). Tobias & Herreid (45) used the 2,4-dinitrophenylhydrazine method to investigate factors (temperature, copper, light) affecting the oxidation of ascorbic acid in milk to dehydroascorbic acid, thence to 2,3-diketo-l-gulonic acid and the subsequent further destruction of the gulonic acid. The losses of reduced ascorbic acid during bulk storage of milk at 38–49° F for 36 h has been studied (46). The ascorbic-acid contents of concentrated sterilized milk before and after 6 months storage at 10–15 °C were 13 and 7 μg/kg (47). Sweetened condensed milk had an ascorbic-acid content of 22·6 mg/kg; fortification with 200–500 mg of ascorbic acid per kg of condensed milk before canning gave a product that still contained 154–424 mg/kg after storage at room temperature for 9 months; during subsequent storage of up to 42 months the vitamin losses were small and no significant deterioration in the quality of the product was noted (48). Raising the storage temperatures from 20 to 40 °C increased the rate of destruction of sodium ascorbate added to evaporated milk (49). Kankrej cows secreted colostrum and milk containing 3·4 and 1·8 mg/100 ml of ascorbic acid (50). Daily oral dosing of cows and buffaloes with 8 mg of L-thyroxine reduced the amount of ascorbic acid in the milk of both species (51).

Ewe's milk in Russia contained 100 μg of thiamine and 280 μg of riboflavin per 100 ml (52), but in Japan it contained 28 μg of thiamine, 160 μg of riboflavin and 4 mg of ascorbic acid per 100 ml (53); a similar value for ascorbic acid was reported from Yugoslavia (54). The vitamin B_{12} content of the milk of ewes grazing marginally cobalt-deficient pastures in New Zealand averaged 2·5 μg/l., but after they had received pellets containing 90 % of cobaltic oxide the level rose to 10·3 μg/l. (55). The average vitamin B_{12} content of about 200 milk samples from various farm animals in Russia was highest for ewes (7 μg/l.) and lowest (0·38 μg/l.) for goats; the content was lower in samples from areas where the soil had a low cobalt content (56).

Goat's milk in Japan contained 290 μg/100 ml of riboflavin (36). The ascorbic-acid content of goat's milk decreased with increasing age of the animal (57).

The riboflavin and thiamine contents of mare's milk were related to the diet, but were independent of the age of the animal (58,59).

The ascorbic-acid content of buffalo's milk in Russia varied widely from less than 0·2 to 3·2 mg per 100 ml and was not related to feeding or the stage of lactation (60).

Thiamine in human milk is reviewed briefly (61). Values for the riboflavin content of colostrum and milk of nursing mothers in Japan are reported (62). The folinic-acid content of human colostrum and milk decreased throughout lactation from a mean of 650 to 320 mμg/100 ml in the 7th month (63); pantothenic acid increased from 75 μg/100 ml in colostrum to a mean value of 210 μg/100 ml during the 7 months of lactation (64).

Yoghurt contained average riboflavin, biotin and nicotinic-acid concentrations of

177, 1·2 and 186 μg/100 g (65). The vitamin B_{12} content of milk as measured with *Euglena gracilis* decreased during the preparation of yoghurt and acidophilus milk; the final products had contents ranging from 0·017 to 0·083 and from 0·12 to 0·24 μg/100 g solids (66) (cf. (88) and p. 328).

The concentrations of thiamine, riboflavin and nicotinic acid in laboratory prepared vitamin-free casein were 0·04, 1·5 and 1·4 μg/g, respectively. Values for commercial vitamin-free casein showed ranges of 0·02–0·35, 0·14–9·3 and 0·16–6·5 μg/g (67).

Further values for the vitamin content of various cheeses include determinations of vitamin B_6 (40, 68), vitamin B_{12} and folinic acid (69), folic acid (70), vitamin B_{12} in hard cheeses (71), and a study of the losses of riboflavin, nicotinic acid, thiamine and ascorbic acid during the preparation and ripening of Gruyère cheese (72).

The average concentrations of nicotinic acid and riboflavin in samples of Egyptian Kishk were 3·5 and 0·27 mg/100 g (73). Kushuk, an Iraqi fermented milk-wheat product, contained 0·05 mg of riboflavin, 0·21 mg of thiamine and 3·5 mg of nicotinic acid per 100 g dry weight (74).

Riboflavin

Continuing their study of the form of riboflavin in milk, Modi, Owen & Darroch (75) measured the partition of riboflavin between the protein-bound and free forms in the milk of six cows, three of which were injected daily with 6·2 mg of L-thyroxine. Initial total riboflavin values ranged from 0·86 to 1·09 μg/ml and declined by 0·003 to 0·03 μg/ml per day as lactation advanced. Although thyroxine increased the milk yield and heart rate and prevented an increase in phosphatase in the milk it did not affect the concentration or partition of riboflavin. This finding is in contrast to the effect of thyroxine in increasing the proportion of protein-bound thiamine in milk (76).

The occurrence in cow's but not in human milk of a flavokinase synthesizing flavin mononucleotide (FMN) and in both milks of an enzyme converting FMN to flavin adenine nucleotide has been briefly reported (77).

Species differences in the occurrence of xanthine oxidase in milk have been studied (78); xanthine oxidase was not found in mare's, human or sow's milk but cow's, goat's, ewe's and rabbit's milk contained 0·08–0·56, 0·10–0·35, 0·1 and 0·18–0·24 units. Milk from goats dosed with sodium tungstate contained only 0·003 units, probably owing to interference with the uptake of molybdenum (78, 79). The xanthine oxidase activity of cow's milk was found to be proportional to the amount of molybdenum in the milk, which depended on the amount of molybdenum ingested. Feeding inorganic molybdenum increased the content of the metal in milk, but not the xanthine oxidase level if this was relatively high already (80). The role of xanthine oxidase in the development of oxidized flavours in milk has been studied (81).

Vitamin B_6

Although it now seems clear that the loss of vitamin B_6 during heat processing of milk products is not a primary causal factor in precipitating convulsive seizure in infants, interest continues in establishing the extent and nature of the changes that occur. Condensed and spray-dried milk showed losses of 25–30% as compared with raw milk (82, 83). Sterilization of a milk product to which pyridoxal had been added for infant feeding caused a loss of about 23% which was dependent on the length

and the temperature of heating (84): the stability of pyridoxal in such products was not significantly influenced by the type of fat that they contained (85). Gregory (86) has carried out a detailed study of the pyridoxine, pyridoxamine and pyridoxal contents of raw human, goat's and cow's milk and processed cow's milk, as determined by parallel assays with *Saccharomyces carlsbergensis*, *Streptococcus faecalis* R and *Streptococcus faecium*, and *Lactobacillus casei*. In raw cow's milk about 80 % of the vitamin B_6 activity was contributed by pyridoxal and the remainder by pyridoxamine. Evaporated milk contained only 40 % of the activity of the raw milk, mainly through loss of pyridoxal. Further losses occurred on storage at room temperature of evaporated milk and the residual activity was due to a substance other than pyridoxal or pyridoxamine, possibly pyridoxine. Samples of these milks were also assayed in biological tests (87); the vitamin B_6 activities determined by microbiological, chick and rat assays and expressed as μg pyridoxine/g of freeze-dried milk were 3·4, 3·2 and 4·9 for raw, 1·0, 2·1 and 2·7 for evaporated and 0·6, 1·4 and 2·0 for stored evaporated milk. The difference between the biological and microbiological tests was statistically significant for the processed milk and the reasons for this difference are being further studied.

Vitamin B_{12}

Callieri (88) has made a detailed study of the distribution of free and bound vitamin B_{12} in milk and milk products. He confirmed the earlier finding (89) that cyanocobalamin is bound in raw milk and showed that the binding capacity is lost on heating milk to 50–60 °C and that such heating releases some cyanocobalamin from the complex. Thus, whereas raw milk contained 3–6 mμg of bound cyanocobalamin per ml, milk pasteurized at 74 °C for 16 sec contained a total of 2·5–5·3 mμg of cyanocobalamin per ml of which 0·14–0·6 mμg/ml was free. Studies of the vitamin B_{12} content of sour milk and yoghurt showed that both contained very much less cyanocobalamin than the milk from which they were prepared. This indicated that the vitamin is used by the bacteria during the fermentation of the milk. Both fermented products and some cheeses contained appreciable amounts of methionine and deoxyribosides, so care is needed in interpreting the results of microbiological assay of the vitamin B_{12} potency of these products.

MINERALS

General

The calcium requirements of humans are discussed in a stimulating review (90), and calcium requirement and adaptation in adult man in an excellent monograph (90*a*).

The role of lactose in enhancing the uptake of calcium has been further studied by Lengemann, Wasserman & Comar (91, 92) who showed that lactose increased ^{45}Ca absorption when this was given to rats as the chloride, gluconate, lactate or acetate. The effect was also found in rachitic rats and was independent of the presence of vitamin D. They showed that the effective site of lactose action was the small intestine, particularly the ileum, and that the presence of lactose in other body tissues had no effect (93). As the effect was not influenced by antibacterial agents it seemed that the mechanism was not dependent on bacterial growth. Fournier &

Dupuis (94, 95, 96) also confirmed the enhancing effect of lactose which they found was greater with rats receiving a calcium-deficient rather than a normal diet.

Topical interest in the radionuclide content of milk which results from contamination of pastures and herbages by the fall-out of radioactivity from nuclear weapon tests has prompted a number of studies of the relation between the level of contamination and factors such as nature of the herbage, geographical situation and climatic conditions. Present indications are that ^{90}Sr levels in milk are still increasing in most parts of the world.

A method for the rapid ashing of milk for radionuclide analysis has been described (97) and the method used for determining ^{89}Sr, ^{90}Sr, ^{140}Ba and ^{137}Cs in milk ash (98). Ion exchange resins may be used to remove from milk Sr and Ca (99, 100), Sr and Cs, without altering the ratios Ca:K and Ca:Na (101), and Sr (102) and ^{131}I (102, 103). Determinations of radionuclides in milk and milk products include values for ^{90}Sr (104, 105, 106), and ^{89}Sr and ^{137}Cs (106) in the United Kingdom; for ^{90}Sr (107, 108, 109), ^{89}Sr, ^{140}Ba, ^{137}Cs and ^{131}I (109) in the United States; for ^{89}Sr and ^{90}Sr in France (110); and for ^{90}Sr in Germany (111, 112), Switzerland (113) and Australia (114). Problems relating to the uptake of strontium by pasture plants are discussed in (115, 116). The transfer from diet to milk of ^{45}Ca and ^{90}Sr has been studied (117) and of ^{89}Sr and ^{131}I (118).

Selenium and the necrogenic properties of dried skim-milk

During the last few years Fink and his collaborators have reported a series of experiments which demonstrated that a large proportion of samples of roller-dried skim-milk, and some spray-dried powders, caused liver necrosis in rats under conditions in which fresh milk did not (119, 120, 121, 122, 123, 124). The albino rats used in these experiments received a diet containing 10 % of milk protein which constituted over 90 % of the protein of the diet and they remained on the experimental diet for up to 120 days. Rats fed on the diets containing dried skim-milk grew normally until shortly before death which was due to liver necrosis. The overall mean death rate was 76 % with forty-nine samples of roller-dried skim-milk and 40 % with eleven samples of spray-dried skim-milk (125). Animals fed the diet prepared with fresh skim-milk grew and remained healthy throughout the experiment. An attempt was made to discredit Fink's experiments by Lang, Pabst & Griem (126) who carried out similar experiments using protein levels of 5–12 % and showed that the incidence of liver necrosis was inversely related to the protein level of the diet. Their findings were rightly rebutted by Fink (127) who had found the same thing himself (128, 129) and who pointed out that the process of drying clearly caused some change in the nutritive value of the milk as fresh milk did not produce the effect when fed to supply the same level of protein. Neither preheating at 85–88 °C for 1 h, sterilizing at 120 °C for 1½ h, nor drying at 146° C caused the injury; it occurred only when preheating was later followed by drying (125, 130, 131).

Fink first believed that the necrogenic properties of dried skim-milk stemmed from damage to some essential amino acids (122). However, in 1957 Schwartz (132) showed that sources of Factor III (133), a dietary agent that prevents liver necrosis in rats, could be replaced by inorganic compounds of selenium. Subsequently Fink (134, 135) showed that a daily dose of 0·4 μg of sodium selenite prevented the onset of liver

necrosis in rats receiving a roller-dried skim-milk diet that caused necrosis in all of a group of six rats not receiving selenium.

The next step clearly is to determine the concentration of selenium in milk and to investigate the apparent loss of availability of selenium during the drying of milk.

MILK AND MILK PRODUCTS FOR THE PRE-RUMINANT CALF

Energy, protein, mineral and vitamin requirements of calves have been reviewed (136) as have the utilization of fats, carbohydrates and proteins by calves (137). The economics of different feeding methods in calf rearing have been considered (138).

The usual daily allowance of whole milk for young calves is 1 lb of milk per 10 lb of body weight. Roy, Shillam, Hawkins & Lang (139), pointing out that such an allowance does not give the small calf the opportunity for as high a rate of growth as the large, have reassessed the milk requirements of the young calf and from extensive trials have derived standards that they consider to be more suitable for experimental purposes than the customary allowance. The protein and energy requirements of the calf have been further investigated (140, 141).

Studies of carbohydrate utilization in the young calf have continued and have confirmed that of a number of carbohydrates tested only glucose, lactose and galactose are utilized (142, 143, 144). Maltose was utilized when given with maltase (142) and sucrose when given with invertase (143). However, attempts to improve the utilization of milk substitutes consisting largely of vegetable products by the addition of proteolytic and carbohydrase enzymes proved unsuccessful (145, 146).

The addition of fat to milk substitute diets provides a cheap source of energy, and diets supplemented in this way allow a high calorie intake for a lower consumption of solids than do carbohydrate-containing diets of similar energy content. Hopkins, Warner & Loosli (147) showed that the addition of lecithin improved the digestibility for 2-week-old calves of coconut oil and tallow added to skim-milk. The utilization, as well as the digestibility of milk substitute diets, was determined by Raven & Robinson who compared nitrogen retention by calves given whole milk with that on diets made from skim-milk with additional carbohydrate or fat. Their experiments showed that hydrogenated (148), but not unhydrogenated (149), palm oil was inferior to butter as a source of energy. The inferiority was partly due to a lower digestibility but mainly to poorer utilization as measured by their relative protein-sparing effects.

A new product has been prepared from whey by fermentation with *Lact. bulgaricus*, followed by the addition of ammonia to neutralize the lactic acid, and concentration to 90 % total solids; the final product contained 0·8 % of nitrogen (150). Feeding tests showed that it was rather unpalatable, but the inclusion of 5 % of it in a milk substitute gave a diet that was consumed by calves, and in which the nutrients appeared to be utilized (151).

Further studies are reported of the value of colostrum (152) and buttermilk (153) in calf rearing; successful trials with formalin-treated skim-milk have been conducted (154).

REFERENCES

(1) Orr, M. L. & Watt, B. K. (1957). *Home Econ. Res. Rep. U.S. Dep. Agric.* **4**, 82 pp. (Washington, D.C.).
(2) Henry, K. M. & Kon, S. K. (1958). *Proc. Nutr. Soc.* **17**, 78.
(3) Bender, A. E. (1958). *Proc. Nutr. Soc.* **17**, 85.
(4) Carpenter, K. J. (1958). *Proc. Nutr. Soc.* **17**, 91.
(5) Platt, B. S. & Miller, D. S. (1958). *Proc. Nutr. Soc.* **17**, 106.
(6) Forbes, R. M., Vaughan, L. & Yoke, M. (1958). *J. Nutr.* **64**, 291.
(7) Henry, K. M. & Porter, J. W. G. (1959). *Proc. XVth Int. Dairy Congr.* **1**, 425.
(8) Causeret, M. J., Hugot, D. & Mocquot, M. G. (1959). *C.R. Acad. Agric. Fr.* **45**, 78.
(9) Randoin, L. & Causeret, M. J. (1956). *Ann. Tech. Agric.* **11**, 535.
(10) Guezennec, J., Girard, P. & Lanteaume, M. Th. (1958). *Lait*, **38**, 369.
(11) Ney, L. F. (1958). *J. agric. Food Chem.* **6**, 223.
(12) Ford, J. E. (1957). *J. Dairy Res.* **24**, 360.
(13) Tomarelli, R. M., Minnick, N., d'Amato, E. & Bernhart, F. W. (1959). *J. Nutr.* **68**, 265.
(14) Diaz, F., Speer, V. C., Homeyer, P. G., Hays, V. W. & Catron, D. V. (1959). *J. Nutr.* **68**, 131.
(15) Dju, M. Y., Baur, L. S. & Filer, L. J., Jr. (1957). *J. Nutr.* **63**, 437.
(16) Tsien, W. S. & Johnson, B. C. (1959). *J. Nutr.* **69**, 45.
(17) Metta, V. C. & Johnson, B. C. (1956). *J. Nutr.* **59**, 479.
(18) Harper, A. E. (1959). *J. Nutr.* **67**, 109.
(19) Gupta, J. D., Dakroury, A. M. & Harper, A. E. (1958). *J. Nutr.* **64**, 447.
(20) Watts, J. H., Booker, L. K., McAfee, J. W., Graham, D. C. W. & Jones, F., Jr. (1959). *J. Nutr.* **67**, 497.
(21) Gupta, J. D., Dakroury, A. M., Harper, A. E. & Elvehjem, C. A. (1958). *J. Nutr.* **64**, 259.
(22) Mauron, J. & Mottu, F. (1958). *Arch. Biochem. & Biophys.* **77**, 312.
(23) Mauron, J., Mottu, F., Bujard, E. & Egli, R. H. (1955). *Arch. Biochem. Biophys.* **59**, 433.
(24) Baur, L. B. & Filer, L. J., Jr. (1959). *J. Nutr.* **66**, 545.
(25) Hutchinson, J. B., Moran, T. & Pace, J. (1959). *Brit. J. Nutr.* **13**, 151.
(26) Howard, H. W., Monson, W. J., Bauer, C. D. & Block, R. J. (1958). *J. Nutr.* **64**, 151.
(27) Block, R. J. & Mandl, R. H. (1958). *J. Amer. diet. Ass.* **34**, 724.
(28) Reussner, G. H., Jr. & Thiessen, R., Jr. (1958). *Food Res.* **23**, 244.
(29) Thiessen, R., Jr. & Reussner, G. H., Jr. (1958). *J. agric. Fd Chem.* **6**, 368.
(30) Kofrányi, E. (1957). *Hoppe-Seyl. Z.* **309**, 253.
(31) Singh, R. & Merilan, C. P. (1959). *Res. Bull. Mo. agric. Exp. Sta.* **688**, 28 pp.
(32) Gregory, M. E., Ford, J. E. & Kon, S. K. (1958). *J. Dairy Res.* **25**, 447.
(33) Ziporin, Z. Z., Kraybill, H. F. & Thach, H. J. (1957). *J. Nutr.* **63**, 201.
(34) Causeret, J. (1959). *Lait*, **39**, 159.
(35) Herrera, C. L. & Albacete, J. A. R. (1957). *Rev. Patron. Biol. Anim., Madrid*, **3**, 303.
(36) Funai, Y. (1955). *Tokushima J. exp. Med.* **2**, 201.
(37) Catellani, G. (1955). *Acta Med. vet.* **1**, 433.
(38) Nani, S. & Redaelli, G. (1958). *Arch. vet. ital.* **9**, 391.
(39) Davidov, R. & Kruglova, L. (1959). *Mol. Prom.* **20**, 40.
(40) Lieck, H. & Søndergaard, H. (1958). *Int. Z. Vitaminforsch.* **29**, 68.
(41) Karlin, R. (1959). *C.R. Soc. Biol., Paris*, **153**, 127.
(42) Hellström, V. (1959). *Vär föda*, **11**, 33.
(43) Antener, I. (1959). *Int. Z. Vitaminforsch.* **29**, 357.
(44) Inikhov, G. & Burenina, E. (1956). *Mol. myasn. Zhivotn.* **1**, 57.
(45) Tobias, J. & Herreid, E. O. (1959). *J. Dairy Sci.* **42**, 428.
(46) Liska, B. J. & Calbert, H. E. (1958). *J. Milk Tech.* **21**, 252.
(47) Sokolov, F. (1957). *Mol. Prom.* **18**, 20.
(48) Sokolov, F. (1959). *Mol. Prom.* **20**, 16.
(49) Conochie, J. (1958). *Aust. J. Dairy Tech.* **13**, 80.
(50) Patel, P. S. & Patel, B. M. (1959). *Indian J. Dairy Sci.* **12**, 27.
(51) Chanda, R. (1958). *Curr. Sci.* **27**, 102.
(52) Davidov, R. B. & Gul'ko, L. E. (1958). *Ovtsevodstvo*, **4**, 23.
(53) Nakanishi, T. & Tokita, F. (1957). *Tohoku J. agric. Res.* **8**, 155.
(54) Panić, B. (1957). *Zborn. Rad. pol'opriv. Fak.* **5**, 121.
(55) Hart, L. I. & Andrews, E. D. (1959). *Nature, Lond.*, **184**, 1242.
(56) Laganovskiĭ, S. Ya. (1958). *Dokl. vses. Konf. moloch Delu*, p. 421.
(57) Ferrara, B. & Minieri, L. (1955). *Acta med. vet.* **1**, 449.
(58) Davidov, R. B. & Gul'ko, L. E. (1958). *Konevodstvo*, **28**, 38.
(59) Bekhova, E. A. (1958). *Dokl. vses. Konf. moloch. Delu*, p. 242.

(60) Akhundov, D. M. (1958). *Dokl. vses. Konf. moloch. Delu*, p. 247.
(61) Anonymous. (1958). *Nutr. Rev.* **16**, 44.
(62) Funai, Y. (1955). *Tokushima J. exp. Med.* **2**, 194.
(63) Karlin, R. (1957). *C.R. Soc. Biol., Paris*, **151**, 1211.
(64) Karlin, R. (1958). *C.R. Soc. Biol., Paris*, **151**, 1521.
(65) Gülmezolu, E. & Físek, N. H. (1956). *Turk. Bull. Hyg. exp. Biol.* **16**, 222.
(66) Emanuilov, I. & Nachev, L. (1957). *Izv. Mikrobiol. Inst. Sofiya*, **8**, 283.
(67) Haenel, H., Wegner, R. & Müller-Beuthow, W. (1956). *Pharmazie*, **11**, 470.
(68) Hathaway, I. L. (1958). *J. Dairy Sci.* **41**, 720.
(69) Karlin, R. (1957). *Ann. Nutr., Paris*, **11**, 91.
(70) Karlin, R. (1959). *Ann. Nutr., Paris*, **12**, 115.
(71) Emanuilov, I. & Nachev, L. (1959). *Izv. Mikrobiol. Inst. Sofiya*, **10**, 57.
(72) Randoin, L. & Causeret, M. J. (1958). *Lait*, **38**, 43.
(73) El-Sadek, G. M., Zawahry, M. R., Mahmoud, S. A. Z. & Abd El-Motteleb, L. (1958). *Indian J. Dairy Sci.* **11**, 67.
(74) Frankul, W. & Pellett, P. L. (1959). *Proc. Nutr. Soc.* **18**, xxxvi.
(75) Modi, V. V., Owen, E. C. & Darroch, R. A. (1959). *J. Dairy Res.* **26**, 277.
(76) Chanda, R., McNaught, M. L. & Owen, E. C. (1952). *Biochem. J.* **51**, 543.
(77) Giri, K. V., Rao, N. A. & Deb, A. K. (1958). *Naturwissenschaften*, **45**, 340.
(78) Modi, V. V., Owen, E. C. & Proudfoot, R. (1959). *Proc. Nutr. Soc.* **18**, i.
(79) Owen, E. C. & Proudfoot, R. (1959). *Proc. Nutr. Soc.* **18**, i.
(80) Kiermeier, F. & Capellari, K. (1958). *Biochem. Z.* **330**, 160.
(81) Aurand, L. W. & Woods, A. E. (1959). *J. Dairy Sci.* **42**, 1111.
(82) Müller, U., Stenger, K. & Wolf, H. (1959). *Milchwissenschaft*, **14**, 115.
(83) Wolf, H. (1958). *Int. Z. Vitaminforsch.* **28**, 281.
(84) Garrett, O. F., Sharp, R. W. & Kleckner, J. (1958). *J. Dairy Sci.* **41**, 711.
(85) Baur, L. S. & Garrett, O. F. (1958). *J. Dairy Sci.* **41**, 711.
(86) Gregory, M. E. (1959). *J. Dairy Res.* **26**, 203.
(87) Davies, M. K., Gregory, M. E. & Henry, K. M. (1959). *J. Dairy Res.* **26**, 215.
(88) Callieri, D. A. (1959). *Acta chem. scand.* **13**, 737.
(89) Gregory, M. E. (1954). *Brit. J. Nutr.* **8**, 340.
(90) Leitch, I. & Aitken, F. C. (1959). *Nutr. Abstr. Rev.* **29**, 393.
(90*a*) Malm, O. J. (1958). *Scand. J. clin. Invest.* **10**, suppl.
(91) Lengemann, F. W., Wasserman, R. H. & Comar, C. L. (1959). *Fed. Proc.* **18**, 733.
(92) Lengemann, F. W., Wasserman, R. H. & Comar, C. L. (1959). *J. Nutr.* **68**, 443.
(93) Lengemann, F. W. (1959). *J. Nutr.* **69**, 23.
(94) Dupuis, Y. (1959). *C.R. Acad. Sci., Paris*, **248**, 1852.
(95) Dupuis, Y. & Fournier, P. (1959). *C.R. Acad. Sci., Paris*, **248**, 2246.
(96) Fournier, P. & Dupuis, Y. (1959). *C.R. Acad. Sci., Paris*, **248**, 1419.
(97) Murthy, G. K. & Campbell, J. E. (1959). *J. Dairy Sci.* **42**, 1288.
(98) Murthy, G. K., Jarnagin, L. P. & Goldin, A. S. (1959). *J. Dairy Sci.* **42**, 1276.
(99) Demott, B. J. & Cragle, R. G. (1959). *J. Dairy Sci.* **42**, 897.
(100) Myers, N. A. (1959). *Nature, Lond.*, **183**, 1807.
(101) Migicovsky, B. B. (1959). *Nature, Lond.*, **184**, 374.
(102) Cosslett, P. & Watts, R. E. (1959). *A.E.R.E.* R 2881, 9 pp.
(103) Glueckauf, E., Cosslett, P. & Watts, R. E. (1958). *A.E.R.E.* CM 371, 2 pp.
(104) Bryant, F. J., Morgan, A. & Spicer, G. S. (1958). *A.E.R.E.* HP/R 2730, 20 pp.
(105) *Agric. Res. Coun. Radiobiol. Lab. Rep.* (1959) **1**, 75 pp. London: H.M.S.O.
(106) Anderson, W., Burton, L. K. & Crookall, J. O. (1959). *Nature, Lond.*, **184**, 89.
(107) Eisenbud, M. (1959). *Science*, **130**, 76.
(108) Kulp, J. L., Slakter, R. & Schulert, R. (1959). *J. agric. Fd Chem.* **7**, 466.
(109) Campbell, J. E., Murthy, G. K., Goldin, A. S., Robinson, H. B., Straub, C. P., Weber, F. J. & Lewis, K. H. (1959). *Amer. J. publ. Hlth*, **49**, 225.
(110) Jeanmaire, L. & Michon, G. (1959). *Lait*, **39**, 369.
(111) Merten, D. & Knoop, E. (1958). *Kieler Milchwirtschaft. ForschBerichte*, **10**, 1.
(112) Heiss, E. (1959). *Dtsch. Molkereiztg.* **80**, 1723.
(113) Miserez, A. (1958). *Mitt. Lebensm. Hyg., Bern*, **48**, 468.
(114) Bryant, F. J., Dwyer, L. J., Martin, J. H. & Titterton, E. W. (1959). *Nature, Lond.*, **184**, 755.
(115) Koontz, H. V. (1959). *Nature, Lond.*, **183**, 1447.
(116) Russell, R. S. (1959). *Nature, Lond.*, **183**, 1806.
(117) Cragle, R. G. & Demott, B. J. (1959). *J. Dairy Sci.* **42**, 1367.
(118) Garner, R. J. & Sansom, B. F. (1959). *Vet. Rec.* **71**, 670.
(119) Fink, H., Schlie, I., Ruge, U. & Grau, A. (1955). *Naturwissenschaften*, **42**, 21.

(120) Fink, H. (1955). *Hoppe-Seyl. Z.* **298**, 93.
(121) Fink, H. & Schlie, I. (1955). *Naturwissenschaften*, **42**, 446.
(122) Fink, H. (1956). *Chemikerztg.* **80**, 4 pp.
(123) Fink, H., Schlie, I. & Ruge, U. (1957). *Naturwissenschaften*, **44**, 283.
(124) Fink, H., Schlie, I. & Ruge, U. (1958). *Z. Naturf.* **13***b*, 610.
(125) Fink, H., Schlie, I. & Ruge, U. (1959). *Milchwissenschaft*, **14**, 572.
(126) Lang, K., Pabst, W. & Griem, S. (1958). *Naturwissenschaften*, **45**, 373.
(127) Fink, H. (1958). *Naturwissenschaften*, **45**, 375.
(128) Fink, H. & Brenner, G. (1957). *Hoppe-Seyl. Z.* **309**, 226.
(129) Fink, H. (1958). *Z. Naturforsch.* **13***b*, 466.
(130) Fink, H., Schlie, I., Herold, E., Brenner, G. & Fink, W. (1957). *Naturwissenschaften*, **44**, 284.
(131) Fink, H., Schlie, I., Ruge, U., Brenner, G. & Herold, E. (1958). *Naturwissenschaften*, **45**, 166.
(132) Schwartz, K. (1957). *J. Amer. chem. Soc.* **79**, 3292.
(133) Schwartz, K. (1951). *Proc. Soc. Exp. Biol., N.Y.*, **78**, 852.
(134) Fink, H. (1958). *Z. Naturforschung*, **136**, 757.
(135) Fink, H. (1959). *Milchwissenschaft*, **14**, 325.
(136) Roy, J. H. B. (1959). *Proc. Conf. Scientific Principles of Feeding Farm Live Stock*, pp. 48–80.
(137) Preston, T. R. (1958). *Proc. Nutr. Soc.* **17**, 63.
(138) Butler, J. B. (1958). *Agric. Rev.* **3**, 38.
(139) Roy, J. H. B., Shillam, K. W. G., Hawkins, G. M. & Lang, J. (1958). *Brit. J. Nutr.* **12**, 123.
(140) Brisson, G. J., Cunningham, H. M. & Haskell, S. R. (1957). *Canad. J. Anim. Sci.* **37**, 157.
(141) Cunningham, H. M., Haskell, S. R., Miles, V. J., Logan, V. S. & Brisson, G. J. (1958). *Canad. J. Anim. Sci.* **38**, 33.
(142) Dollar, A. M. & Porter, J. W. G. (1959). *Proc. XVth Int. Dairy Congr.* **1**, 185.
(143) Okamoto, M., Thomas, J. W. & Johnson, T. L. (1959). *J. Dairy Sci.* **42**, 920.
(144) Velu, J. G., Gardner, K. E. & Kendall, K. A. (1959). *J. Dairy Sci.* **42**, 920.
(145) Fries, G. F., Lassiter, C. A. & Huffman, C. F. (1958). *J. Dairy Sci.* **41**, 1081.
(146) Lassiter, C. A., Fries, G. F., Huffman, C. F. & Duncan, C. W. *J. Dairy Sci.* **42**, 666.
(147) Hopkins, D. T., Warner, R. G. & Loosli, J. K. (1958). *Proc.* 1958 *Cornell Conf. Feed. Manuf.* p. 40.
(148) Raven, A. M. & Robinson, K. L. (1958). *Brit. J. Nutr.* **12**, 469.
(149) Raven, A. M. & Robinson, K. L. (1959). *Brit. J. Nutr.* **13**, 178.
(150) Arnott, D. R., Patton, S. & Kesler, E. M. (1958). *J. Dairy Sci.* **41**, 931.
(151) Hazzard, D. G., Kesler, E. M., Arnott, D. R. & Patton, S. (1958). *J. Dairy Sci.* **41**, 1439.
(152) Wing, J. M. (1958). *J. Dairy Sci.* **41**, 1434.
(153) Snook, L. C. (1958). *J. Dep. Agric. W. Aust.* **7**, 319.
(154) Evans, W. M. R. & Vale, T. W. (1958). *J. agric. Soc. Univ. Coll. Wales*, **39**, 27.

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